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Hsp47 is a chaperone protein with a fundamental role in the folding, stability and intracellular transport of procollagen triple helices. A light-responsive Hsp47 recombinant protein, engineered to control in situ the production and assembly of cellular collagen is here demonstrated. This novel light-driven tool enables unprecedented fundamental studies of collagen biosynthesis and associated diseases.

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Photoactivatable Hsp47: A tool to control and regulate collagen secretion & assembly

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Abstract: *Collagen is the most abundant structural protein in mammals and plays a major role for the mechanical integrity of tissues. It forms supramolecular assemblies with tightly controlled morphologies at different length scales. Alterations in the expression and folding of collagen have drastic implications on the organization of the extracellular matrix (ECM) and the mechanical properties of tissues and result in various collagen-related pathologies, like fibrosis, cancer. Hsp47, a 47 kDa, endoplasmic reticulum resident collagen specific chaperone, plays a crucial role in collagen synthesis, assembly and organization and has been proposed as therapeutic target in collagen-related diseases. In this work, we have created a derivative of Hsp47 that allows spatiotemporally regulation of collagen biosynthesis within mammalian cells using light. A photoactivatable Hsp47 was developed by mutating 383Tyr position at the collagen binding site of the native protein by the light-responsive o-nitro benzyl tyrosine (ONBY). In the mutated form, the function of the protein is arrested. Light exposure activates protein and restores Hsp47 function. The photoactivatable protein was successfully delivered specifically to the endoplasmic reticulum via retrograde KDEL receptor mediated uptake. Using this tool, we demonstrate the possibility of photo regulation of collagen assembly by in situ increasing Hsp47 concentration in the cell upon light exposure. Light-mediated collagen production at post-translational level and subsequent ECM remodelling are demonstrated. The ability of photoactivatable Hsp47 to promote collagen synthesis in diseased state cells on-demand is shown. This tool will allow unprecedented fundamental studies of collagen biosynthesis, help in inspiring new therapeutic concepts in biomedicine and tissue regeneration.*

Collagen is a major component of the extracellular matrix, and a key structural protein for the mechanical integrity of tissues[1]. Collagen biosynthesis and the formation of collagen-characteristic superstructures involves a complex orchestration of intracellular and extracellular chemical and assembly events[2] regulated by specific enzymes[2] and molecular chaperones, and assisted by different enzymes and folding modulators[1-3]. The collagen triple helices self-assemble to form fibrils, beaded filaments or networks depending on the collagen type, and give rise to different tissue architectures and derived tissue-specific mechanical properties: rigid bone, compliant skin, or gradient mechanics in cartilage tissue[4]. Changes in the synthesis and assembly of collagen are prevalent in pathologies like Osteogenesis Imperfecta, Ehlers-Danlos Syndrome and Epidermolysis Bullosa [5]. Collagen is also the subject of scrutiny in pathologies like fibrosis and tumor formation[3e, 6]. While collagen folding and assembly is of vital relevance, very few tools exist to probe the intricacies of the various processes involved,[7] and the mechanisms involved are far from been understood.

Hsp47 (Heat shock protein 47) is a chaperone protein with a fundamental role in the folding, stability and intracellular transport of procollagen triple helices. It is a highly conserved,47-kDa endoplasmic reticulum (ER) resident protein and binds to collagen of at least types I to V [8]. Most interestingly, in striking contrast to other chaperones, it is specific for collagen and it preferentially recognizes the folded triple-helical conformation of its client[9]. Hsp47 stabilizes the collagen triple helix, which is inherently unstable at physiological temperature, and protects it from intracellular degradation. It provides a quality control mechanism for correct helical folding and assembly and prevents premature aggregation of procollagen molecules in the ER into large collagen aggregates, difficult to transport[9a]. The highest-affinity sites for Hsp47 are located in the N-terminal region of procollagen. Since triple-helix formation occurs in a zipper-like fashion, proceeding from the C- to the N-terminus, binding of Hsp47 to folded segments at the N terminus could signal a successfully terminated folding event and mark this complex for passage to the Golgi. This is in agreement with the finding that Hsp47−/− cells secrete collagen much more slowly and are deficient in N-propeptide processing[3b]. When collagen-bound Hsp47 is carried to the Golgi body via vesicular transport, the KDEL sequence at its C-terminus is recognized by the KDEL receptor, which retrieves it and returns it to the ER [10].Artificial removal of this sequence results in the secretion of the protein from the cell by exocytosis[11].

Hsp47 exhibits the typical serpin fold, consisting of three β-sheets (A, B, and C) and nine α helices and does not change its conformation upon collagen binding. There are six histidine residues on Hsp47 that are located in the vicinity of the collagen–Hsp47 interface: His215, His216, His238, His273, His274, and His386. These are responsible for the stabilization of the Hsp47-Collagen complex at neutral pH in the ER. Upon arrival at the Golgi apparatus, the acidic pH medium mediates protonation of Hsp47’s histidine’s and assists the release of Hsp47 from procollagen[3d, 10b, 12]. Hsp47 is specifically expressed by cells that synthesize large amounts of collagen. It has a vital role as shown by the embryonic lethal phenotype of Hsp47 knockout mice[13]. Constitutive expression levels of Hsp47 correlate strictly with the amounts of collagen being synthesized in the corresponding cells[13b, 14]. Collagen biosynthesis therefore strongly depends on the correct expression and function of Hsp47, and hence can be altered by interfering with Hsp47 expression. In fact, alterations in Hsp47 expression levels or mutations in Hsp47 correlate with pathological states[5e, 13a]. For example, expression of Hsp47 is up-regulated during the progression of various fibrotic lesions[15],[10b, 13a, 16]. Studies have also shown that suppression of Hsp47 expression can reduce accumulation of collagens and can delay the progression of fibrotic diseases in experimental animal models[17]. In a different context, several reports indicate that Hsp47 is closely linked to different types of Osteogenesis Imperfecta [10b, 13a, 16]. Recently enhanced expression of Hsp47 has been found in cancer tissue [3e, 6a, 6b]. Silencing of Hsp47 expression reprogrammed breast cancer cells to form polarized and/or non-invasive structures in 3D culture, which significantly inhibited tumor growth *in vivo* and reduced deposition of collagen [18]. These results indicate that Hsp47 is relevant for cancer progression, and may represent a potential biomarker and therapeutic target.

The specificity of Hsp47 for collagen, its interdependence with collagen expression, and its crucial role for collagen folding make it an ideal tool to manipulate and study collagen production and assembly at early stages of the biosynthesis process. This idea has motivated the current study, in which we describe a photoactivatable variant of Hsp47 and demonstrate the possibility to remotely activate the biosynthesis of collagen within cells using light. The photoactivatable Hsp47 was recombinantly synthesized in *E. coli* and contained one non-natural light-responsive tyrosine (o-nitro benzyl tyrosine – ONBY) replacing the 383 Tyr at the collagen-binding site[19]. This position was selected based on results from collagen binding assays which have been shown that mutations in 383Tyr lead to complete loss of binding activity[3c, 10, 12, 20]. The 383Tyr codon was replaced with an amber stop codon (TAG), and a previously developed amino acyl tRNA synthetase (aaRS) that couples corresponding tRNA for ONBY was introduced[5a, 21]. The recombinant photoactivatable Hsp47 ($H_{47Y<ONBY}$) was successfully delivered into the ER of fibroblasts by simple incubation via a KDEL receptor mediated endocytosis mechamism. Cells treated with $H_{47Y<ONBY}$ showed light-estimulated production of collagen, which could be controlled in time and space. We envision that photoactivatable Hsp47 will allow

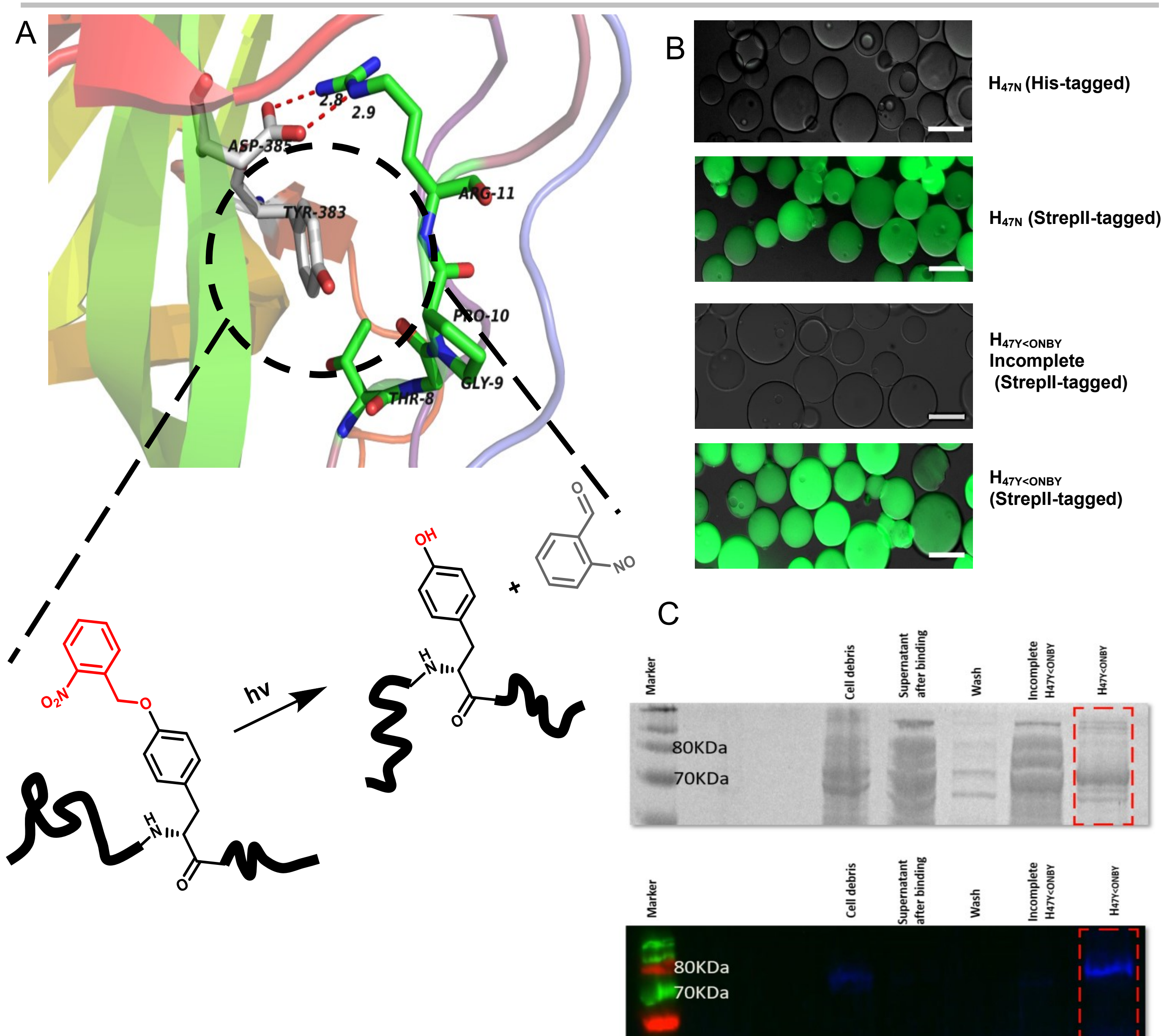

Figure1.**A.**Scheme showing photocleavage of ONBY in Hsp47(Structure of Hsp47:Collagen peptide interaction adpated from Widmer et.al,PNAS 2012). **B.**Microscopy images (merged phase contrast and epifluorescence green channels) of Strep-II-Tagged agarose beads incubated with supernatant of Hsp47 variants after harvesting *E. coli* (Scale bar:100µm) demonstarting complete Photoactivatable Hsp47 having StrepII-tag . **C.** 12 % SDS Page gel of $H_{47Y<ONBY}$ and Western Blot showing the labeling of StrepII-tag at the C-terminus with Alexa 488 conjugated Streptavidin(indicated in blue color)(Molecular weight- 71.46 KDa).

unprecedented fundamental studies of early stages of collagen assembly. Moreover, it might also inspire and support new therapeutic approaches in collagen-related diseases.

We employed a previously established bacteria engineering strategy to site-specifically incorporate the photoactivatable non-natural amino acid *o*-nitrobenzyl *tyrosine (ONBY)* [19, 21a, 22] (see details in Fig S1 at the Supporting Information) into Hsp47 to replace 383Tyr. A previously developed Hsp47 gene coding for residues 36-418 was used.[3d, 10b] In order to improve Hsp47's solubility for heterologous expression in *E. coli*, an enhanced green fluorescent protein (EGFP) was genetically fused to its N-terminal[23]. This approached allowed us to significantly increase the synthesis yield (Table S1). A StrepII-tag was introduced at C-terminal for affinity purification. This derivative of Hsp47 was successfully obtained in 207.8 µg yield from 200mL culture, as confirmed by absorbance with UV-Vis spectrophotometer, and was named H_{47N} (N refers to native). In the synthesis of the photoactivatable version of $H_{47N,}$ an amber codon mutation TAG incorporated ONBY at the 383rd position. This Hsp47 derivative was named $H_{47Y<ONBY}$. In order to confirm the incorporation of ONBY, synthesis variants in the presence or absence of ONBY in the medium before induction were performed. In the presence of ONBY, the full protein would be expressed, whereas in the absence of ONBY translation should terminate at the 383rd position and the strepII-tag would not be included.

StrepII-tagged coated agarose beads were incubated with small amount of cleared lysates and purified Hsp47 control variants. The agarose beads became fluorescent within 5 mins when incubated $H_{47Y<ONBY}$, indicating that the StrepII-tag had been incorporated at the C-terminus (ONBY present) whereas no fluorescence was observed in truncated version having no StrepII-tag (Fig 1B). The clear lysates were purified

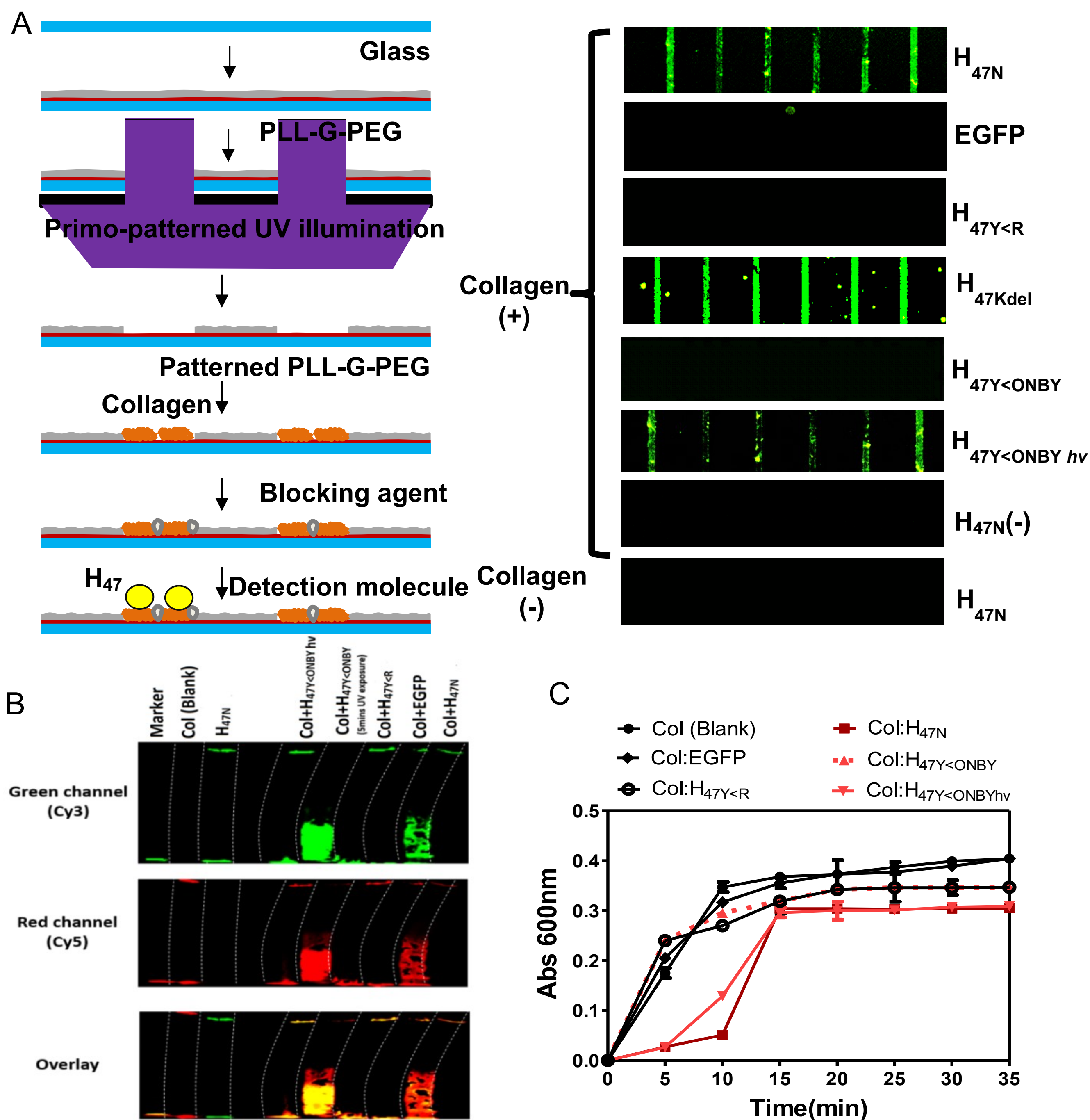

Figure2. **A.** Schematic of binding assay of Hsp47 and its variants to collagen micro-patterns and fluorescence image of micro-patterning protein-protein affinity assay. **B.** Native PAGE Western Blot of rat tail Collagen Type 1 (200ug/mL, 0.6uM) mixed with H_{47N}, $H_{47Y<R}$, $H_{47Y<ONBYhv}$ or H_{47Kdel} (1.2μM) ,demonstrating co-localization of Hsp47 variants with collagen in binding assays. **C.** Fibrillogenesis assay by turbidimetry measurements of collagen (200ug/mL, 0.6uM) mixed with H_{47N}, $H_{47Y<R}$, $H_{47Y<ONBYhv}$ or H_{47Kdel} (1.2μM) at molar ratio 2:1(Hsp47: Collagen) at OD600 values

using StrepII tag purification and screened to affirm the presence of StrepII-tag. Western Blot was performed labeling StrepII-tag using Alexa488 Streptavidin after affinity purification which showed a clear fluorescent band when ONBY was present during synthesis, and no fluorescence was observed in the truncated version (Fig 1C). This result confirmed the incorporation of ONBY to $H_{47Y<ONBY}$. The yield of the synthesis was 43.56ng from 200ml culture.Two additional mutants of H_{47N} were developed as control proteins for further experiments: (i) $H_{47Y<R}$, with Tyrosine mutated to Arginine at 383 position as an inactive version of H_{47N}[16c]. (ii) $H47_{Kdel}$ where the KDEL sequence at C terminus of H_{47N} was deleted, thereby preventing retrograde delivery of the protein into the ER. The yields of all the variants are included in Table S1.

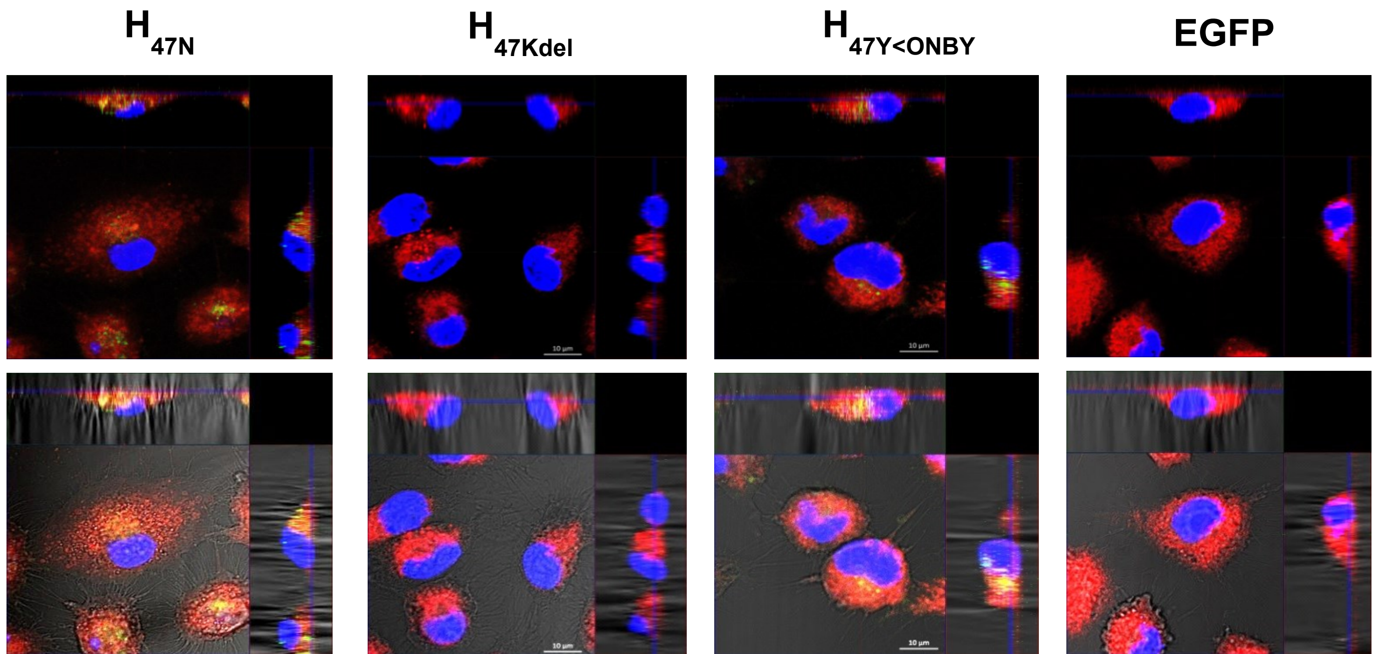

Figure3.Confocal Z stack images of L929 cells after incubation with Hsp47 variants showing colocalisation of H_{47} or $H_{47Y<ONBY}$ signal and ER staining. No fluorescence was observed after incubation with EGFP or H_{47Kdel} constructs (Blue: DAPI (Nucleus), Green: EGFP (Hsp47 variants) and Red: ER tracker Dye) Scale bar:50µm.

In order to proof the affinity for collagen of the different Hsp47 variants (H_{47N}, $H_{47Y<ONBY}$, $H_{47Y<R}$, H_{47Kdel}), the obtained proteins were incubated with collagen I micropatterns (see experimental section for details)[24]. Their interaction with the surface bound collagen was imaged by Nikon Ti-Eclipse microscope. H_{47N} and H_{47Kdel} showed fluorescence bands (green fluorescence due to EGFP tagged constructs) indicating interaction to collagen, whereas $H_{47Y<ONBY,}$ negative control $H_{47Y<R,}$ and the EGFP did not show collagen binding (Fig 2A). Light irradiation of the $H_{47Y<ONBY}$ solution during incubation (*in situ* activation upon 10 sec exposure at 365 nm) lead to appearance of fluorescence. This result demonstrates that photochemical activation of $H_{47Y<ONBYhv}$ renders functional Hsp47, able to bind to collagen. Native PAGE-western blot analysis confirmed these results. Co-localization of fluorescent antibody labeled bands of collagen and H_{47N} or light-activated $H_{47Y<ONBYhv}$ was observed.Coversely, $H_{47Y<ONBY}$, $H_{47Y<R,}$ H_{47Kdel} and EGFP did not co localize with the collagen band,demostarting no collagen binding.
Hsp47 has been shown to prevent collagen fibrillogenesis *in vitro*[10a] .The possibility to control fibrillogenesis *in vitro* using light and $H_{47Y<ONBYhv}$ was tested in a fibrillation assay using turbidity measurements. Previous reports have shown that addition of Hsp47 in 2-fold molar excess to a 0.6 µM collagen solution in PBS delays collagen fibrillation at 34°C[10a, 15] .These results were reproduced with H_{47N} solutions (see Fig S3). When similar experiments were performed in minimum essential medium (MEM), improved gelation was observed confirmed by rehology measurements(S4) which indicates proper collagen fibrillogenesis. This medium is considered more representative of physiological conditions for collagen association[25]. These experiments were also performed with Hsp47 variants at concentrations between 0.1 and 1.2 (data not shown). $H_{47Y<ONBY,}$ $H_{47Y<R}$ and EGFP did not show any effect on fibrillation kinetics of collagen solutions at any of the tested concentrations. Light-activated $H_{47Y<ONBYhv}$ reduced the rate of collagen fibrillation to a similar extent to H_{47N} at comparable concentrations (Fig 2C). These results indicate that $H_{47Y<ONBYhv}$ enables light-triggered interference with lateral association of collagen triple helices and delay of fibril formation.
Hsp47 is an ER resident protein with a C-terminal KDEL retention motif. This motif is recognized by KDEL receptor after Hsp47 release in the Golgi and is responsible or its retention in the ER.[26] In fact, deletion of KDEL sequence has been shown to block the retention of Hsp47 into the ER.[27] The KDEL receptor is also present in the plasma membrane of cells, and has been shown to assist internalization of KDEL containing molecules from the extracellular space[28].We tested if our Hsp47 variants could be delivered to ER using KDEL receptor mediated endocytosis (Fig.3). For this purpose, L929 and MEFs fibroblasts were incubated with Hsp47 variants for 3 h and imaged. Cells incubated with $H_{47N,}$ $H_{47Y<R}$ and $H_{47Y<ONBYhv}$ showed colocalization of the EGFP signal (green in Fig.3) with the ER tracker dye (red),indicating successful uptake of the Hsp47 variant by the cells (see 10X-magnified images in Fig S7). Neither uptake of the H_{47Kdel} variant with deleted KDEL, nor uptake of EGFP was observed after 3 hrs incubation. In order to optimize the Hsp47 concentration for efficient delivery to fibroblast cells, experiments with concentrations of H_{47N} in the incubation medium between 0.01µM and 1µM were performed. 0.2-0.3µM concentration proved to be the best condition for the delivery of the recombinant constructs (see Fig S6). Protein concentrations above 0.3µM resulted in the formation of protein aggregates on the cell culture substrate. These results demonstrate that the photoactivatable $H_{47Y<ONBYhv}$ can be simply introduced into the ER of cells by short incubation, allowing easy experimental implementation of this tool for the study of Hsp47-specific roles in cellular pathways and collagen assembly.
Collagen biosynthesis is dependent on the expression level of functional Hsp47. To determine the bioactivity of uptaken Hsp47 variants, we investigated collagen biosynthesis of Hsp47 -/- cells after incubation with the recombinant proteins. The deposited collagen was quantified using the Sirus Red assay whose dye binds specifically to the [Gly-X-Y] n helical structure on fibrillar collagen (type I to V) [29]. Hsp47 −/− cells incubated with H_{47N} showed a significant increase in collagen production (approximetely 15-20%). Hsp47 −/− cells incubated with $H_{47Y<ONBY}$ did not show increase in collagen production, in agreement with the lack of biofunctionality of $H_{47Y<ONBY}$ observed in previous experiments. Exposure of $H_{47Y<ONBY}$ treated cells with 365 nm light *in situ* activated lead to increase in collagen production. These results demonstrate

biofunctionality of $H_{47Y<ONBY}$ in response to light activation (Fig.4). Ascorbate (Vitamin C) is a widely used chemical inducer for collagen production at translational level by triple helix stabilization for *in virto* studies[13b, 14b, 29-30]. We compared collagen deposition in Hsp47 -/- cells treated with H_{47N}, light exposed $H_{47Y<ONBY}$ and with ascorbate. Interestingly, we found higher levels of collagen production in H_{47N} and light exposed $H_{47Y<ONBY}$ incubated cells compared to ascorbate (Fig.4). Addition of ascorbate to cells containing H_{47N} or light exposed $H_{47Y<ONBYhv}$ had a synergistic effect for collagen production (see fig S5). When Hsp47 +/+ cells were treated with H_{47N} and *in situ* activated $H_{47Y<ONBYhv}$ only a slight increase in collagen production was observed (see fig S8).This might be due to saturation levels of Hsp47 in the ER. Interestingly, proliferation levels in Hsp47 −/− cultures treated with H_{47N} or *in situ* activated $H_{47Y<ONBY}$ slightly increased vs. non treated cultures (see Fig S5). Together, these results demonstrate the efficiency of exogenous $H_{47<ONBY}$ to upregulate collagen biosynthesis upon light exposure (Fig.4).

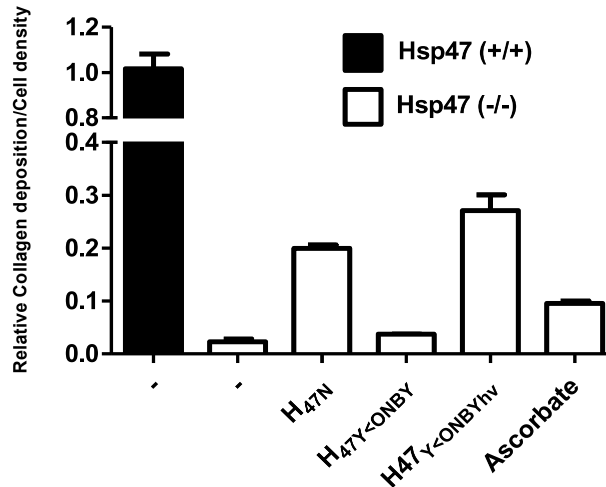

Figure.4.Relative collagen deposition in MEF Hsp47 +/+ and MEF Hsp47 -/- cultures treated with Hsp47 variants and sscorbate. Collagen deposition was calculated using quantified data of Sirus Red Assay at 570 nm.

Finally, we investigated the potential of $H_{47Y<ONBY}$ for spatiotemporal control of collagen production after *in situ* photoactivation of cell cultures. For this purpose an assay for imaging collagen deposition on the culture substrate was established. Hsp47 −/− cells were incubated with the different Hsp47 variants for 3 h. Then the medium was exchanged and cells were cultured for further 24 h, fixed and stained with Sirus Red for imaging deposited collagen Type I on the culture substrates. Hsp47 −/− cells incubated with $H_{47Y<R}$, H_{47Kdel} or EGFP did not show any fluorescence related to collagen production, whereas cells incubated with H_{47N} clear fluorescence signal (Fig 5.B). H_{47N} and light exposed $H_{47Y<ONBY}$ showed EGFP fluorescence at the ER, indicating that the recombinant protein delivered was still present in the cells after 24 hours (Fig 5). This is in agreement with the reported >24 h half-life of Hsp47[31]. $H_{47Y<R}$ did not show EGFP fluorescence after 24 hrs which may be due to degradation of inactive form due to lack of functional activity over time (Fig.5.B). For localized deposition of collagen,180x120mm^2 areas of the Hsp47 -/- cell culture incubated with $H_{47Y<ONBY}$ were irradiated for 30 seconds one hour after medium exchange. Cells at the exposed areas showed significant higher collagen deposition (Fig 5.A) and presence of increase EGFP fluorescence indicates the functional Hsp47 had been activated. The Hsp47-/- and Hsp47 +/+ cells lacking exogenous $H_{47Y<ONBY}$ did not show any increase in collagen staining upon UV exposure, indicating that UV irradiation by itself had no effect on collagen production (Fig.5). These results demonstrate the possibility to photoregulate collagen biosynthesis to produce collagen patterns within cell cultures using $H_{47Y<ONBY}$.

In conclusion, a photoactivatable variant of the collagen-specific protein Hsp47 has been developed by incorporating a photoactivatable Tyr rest at the 383rd amino acid of Hsp47, which is relevant for collagen binding. This recombinant protein could be effectively delivered to the ER of fibroblasts via KDEL receptor mediated endocytosis. *In situ* light exposure allowed light-mediated increase of Hsp47 concentration inside the cells, and consequently localized upregulation of collagen production in cellular cultures. We envision that this tool will allow unprecedented studies of collagen synthesis and early assembly. Moreover, photoactivatable Hsp47 might inspire new therapeutic concepts for treating collagen related defects like Osteogenesis imperfecta, Ehlers-Danlos Syndrome and *Epidermolysis Bullosa*. Finally, the possibility of external upregulation of collagen synthesis might be advantageous for tissue regeneration and rebuilding of the extracellular scaffold.

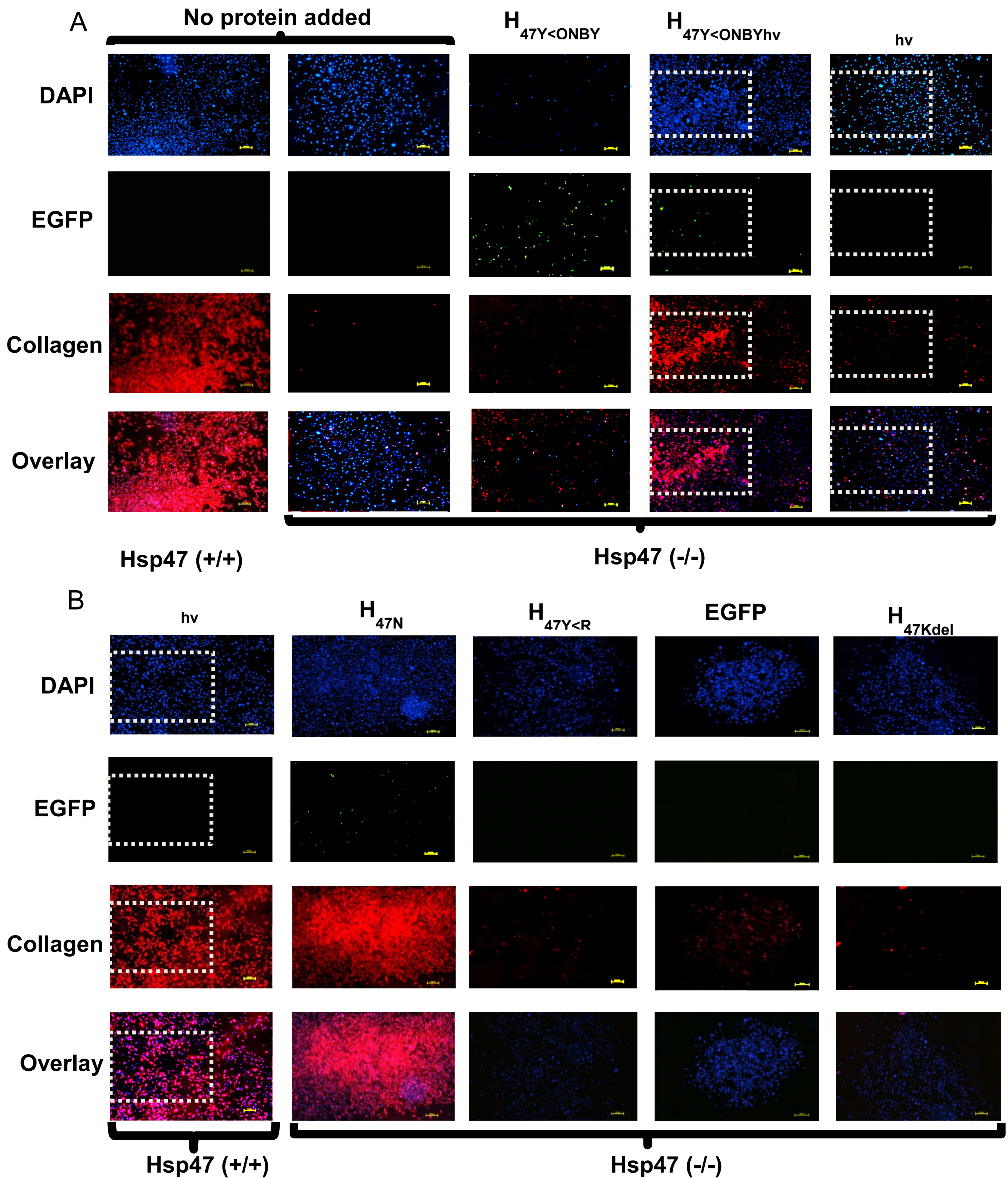

Fig 5. Immunostaining of MEF Hsp47 +/+ and −/− cultures after incubation with Hsp47 variants for 3 h and further culture in MEM for 24 h.The cells were stained with Col1 Antibody (red). Hsp47 variants have green fluorescence and nuclei are stained Blue with DAPI. A.The Hsp47 +/+ and −/− having no protein delivery were used as controls. The light exposed areas are highlighted (180x120 mm^2). Irradiation was done at 365 nm B. Hsp47 +/+ cells incubated with other inactive mutants show no improvement in collagen production whereas the cells incubated with H_{47N} show enhance production of collagen.Scale bar: 250um

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Keywords:
Collagen,Hsp47,Protein Engineering,KDEL Receptor Mediated Endocytosis,Biological activity, Photoactivation,Optoregulation

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Supporting information

Materials and Methods

Cloning and Purification

The psfGFP150TAGPylT-His6pBad plasmid encoding for MbPylT and C-terminal hexahistidine-tagged sfGFP, with an Amber stop codon at position 150 (pBad), and pBK-ONBYRS expressing MbPylS mutant was a gift from Prof. Dr. Jason Chin, Medical Research Council Laboratory of Molecular Biology, University of Cambridge, UK[1]. A construct encoding amino acids 36–418 of the Canine Hsp47 (canine SERPINH1 mRNA, NCBI accession NM_001165888), cloned into the pJExpress vector with a C-terminal Strep II tag was gift from Prof. Dr. Ulrich Baumann, University of Cologne,Germany[2].
The canine-derived synthetic Hsp47 gene from pJExpress plasmid was cloned in pET28a plasmid at the C terminus of EGFP gene using EcoRI and XhoI sites and transformed in NEB5α cells using manufacturer's protocol (NEB C29871). Colonies were picked up and insert screening was performed using colony PCR and confirmed with sequencing following which positively cloned constructs were selected and were further cloned into psfGFP150TAGPylT-His6pBad plasmid at NcoI and XhoI site to develop pEGFPHsp47StrepIITagpBad construct. This construct was used to optimize protein expression conditions and was used as a backbone for developing the final construct pEGFPHsp47TAG 383 StrepIITagpBad construct (described later). Colonies were picked up and insert screening was performed using colony PCR approach and sequencing following which positively cloned constructs were selected and transformed into One shot top10 cells for protein expression using strepTag2 purification. Transformed cells were grown to OD600 of 0.8 in LB medium containing kanamycin (Kan) (25µg/ml) and tetracycline (Tet) (12.5µg/ml). These cells were pelleted and the medium was exchanged in sterile environment with pre-warmed Terrific broth containing kanamycin (25µg/ml) and tetracycline (12.5µg/ml) and protein expression was induced after half an hour with 0.2 % arabinose at 37°C / 250 rpm. After induction, the cultures were incubated for 1 hour at 37°C / 250 rpm following which it was kept at 25-30°C / 180 rpm cells overnight. The cultured cells were harvested and pellets stored at -80 °C. Cells were resuspended in lysis buffer (50 mM Tris ·HCl (pH 7.5), 150 mM NaCl, 100 µ M PMSF, 4 mM DTT) and lysed by sonication. Cleared lysates were loaded onto 1 ml Streptactin Superflow high capacity binding column (IBA) and eluted with 2.5 mM (D)-desthiobiotin in lysis buffer excluding PMSF.
For the expression of photoactivatable Hsp47 ($H_{47Y<ONBY}$), E. coli TOP10 cells co-transformed with pBK-ONBYRS and pEGFPHsp47TAG 383 StrepTagIIpBad (encoding for N terminal EGFP tagged cHsp47 and C-terminal strepIItag with an amber stop codon (TAG) at position 383). The TAG mutation at 383th position was incorporated with NEB Q5 site directed mutagenesis kit using manufacturer's protocol (NEB E0554S).Transformed cells were incubated overnight with shaking at 37°C in LB supplemented with Kan (25µg/ml) and Tet (12.5µg/ml). The overnight culture was diluted 1:100 in two separate volumes of terrific broth (TB) supplemented with the same concentration of antibiotics and incubated with shaking at 37°C. At OD600= 0.8 bacteria were isolated by centrifugation and re-suspended in equal volumes of warm TB supplemented with the same concentration of antibiotics, in the presence or absence of 0.4 mM of O-[(2-Nitrophenyl)methyl]-L-tyrosine hydrochloride (Santa Cruz Biotech). After 30 min, protein expression was induced with the addition of arabinose at a final concentration of 0.2% (w/v). Induction studies were performed with different temperature conditions after induction incubation for 1 hour at 37°C / 250 rpm following with 25-30°C / 180 rpm cells overnight was chosen as optimized expression condition. The cultured cells were harvested and pellets stored at -80 °C. Cells were resuspended in lysis buffer (50 mM Tris ·HCl (pH 7.5), 150 mM NaCl, 100 µ M PMSF) and lysed by sonication. 20µL of the clear lysates were taken incubated with 5 µL of Strep II tagged agarose beads (IBA Life sciences) for 5 min. Previously purified H_{47N} (His-taggged) and H_{47N} (strepIItaggged) were used as controls. The beads were then spun down at 1000 rcf for 30s and washed 2 times with PBS. For purification, cleared lysates were loaded onto 1 ml Strepactin Superflow high capacity binding column (IBA Life sciences) and eluted with 2.5 The bead solutions were placed in 96 well-plate wells and imaged using the Nikon Ti-Eclipse microscope. mM (D)-desthiobiotin in lysis buffer excluding PMSF. The soluble fraction for both H_{47N} and $H_{47Y<ONBY}$ was concentrated and purified further by gel filtration (Superdex S75; GE Healthcare) in buffer A [20 mM Hepes (pH 7.5), 300 mM NaCl, 4 mM DTT]. The strepIItag at C terminus was chosen as a selection marker for ONBY incorporation which was confirmed by Alexa 488 labeled Streptavidin against the strepIItag at the C terminus of $H_{47Y<ONBY}$. The bacteria which were not fed with ONBY in the medium

produced truncated or incomplete $H_{47Y<ONBY}$ because of the TAG mutation. A Western Blot was performed by running protein samples on SDS page. The 12% SDS PAGE gel was transferred using blotting chamber to PVDF membrane. The Blotted PVDF Membrane was blocked with Blocking buffer (0.5% milk powder in PBST (0.1 w/v)) for 20 mins. The excess blocking buffer was washed of three times using PBST (0.1 w/v) and then stained using labeled Fluorescent Alexa 488 streptavidin with a dilution of 1:500 for 20 mins. The excess streptavidin was washed of three times using PBST (0.5 w/v) and image RGB Blot in Gel doc.

In order to have controls for protein and cell based assays the N-terminal E GFP tagged cHsp47 pet28a plasmid were modified to develop variants. The variants were **a.** EGFP-Hsp47 383 Y<R pet28a (encoding $H_{47Y<R}$ with Tyrosine mutated to Arginine at 383th position).**b.** KDEL del EGFP-Hsp47 pet28a (encoding H_{47Kdel} without KDEL sequence at the C terminus). All the mutants were developed using NEB site directed mutagenesis kit (NEB E0554S) and transformed into NEB 5 α cells for plasmid extraction and sequencing analysis. Positive clones were transformed in BL21 (DE3) Clear coli cells for His-tagged Ni-NTA chromatography. Expression was induced in BL21 (DE3) cells grown to an OD600 of 0.6–0.7 by adding 0.5 mM isopropyl-β-d-thio-galactoside and shaking overnight at 25 °C. Cells were resuspended in lysis buffer (300 mM NaCl, 10 mM imidazole, 20 mM Tris, pH 8) and lysed by sonication. Cleared lysate was purified by Ni-NTA affinity chromatography (Ni-NTA superflow; Qiagen). The soluble fraction was concentrated and purified further by gel filtration (Superdex S75; GE Healthcare) in buffer A [20 mM Hepes (pH 7.5), 300 mM NaCl, and 4 mM DTT]. EGFP-pet28a was purified using same approach.

Collagen micropatterned affinity binding assay with Hsp47 variants

Collagen micropatterns were made by adapting a reported protocol[3]. Glass cover-slips were cleaned by 5 min air plasma treatment (Harrick Plasma, Ithaca, NY, USA). In a 20×20x0.25 μm^3 Poly(dimethylsiloxane) (PDMS) membrane having square , nine 3x3 mm^2 wells. The PDMS membrane was placed on the plasma treated cover-slip. In each 3x3 mm^2 well, 5 µL of a 0.1 mg/mL PLL-g-PEG (PLL (20)-g [3.5]-PEG (5), SuSoS AG, Dübendorf, Switzerland) solution in phosphate buffered saline (PBS) was incubated for 1 h. Next, the wells were incubated with 5uL of photo initiator (4-benzoylbenzyltrimethyl ammonium chloride) solution (custom synthesis by Sigma-Aldrich outsourced to SinoChem, China) for 1 minute. A wide field mass less UV projection was employed for making 10 µm patterns for 10 sec having 50%intensity at 365 nm wavelength.The optical projection system is based on a standard epi-fluorescence inverted microscope (Nikon Ti-Eclipse microscope, Nikon Instrument, France) coupled with a Digital Light Processing device (Texas-Instrument DLP Discovery 4100 UV) including a DMD (PRIMO unit) to generate spatially modulated excitation patterns[3].The UV exposure cleaves the PEG chains at the exposed sites on the substrate. After extensive rinsing with PBS, the micro wells were filled with 10 µL of 200ug/mL rat tail collagen I (Fisher Scientific) in H_2O (Diluted from Rat tail collagen stock of 3mg/mL). Collagen adsorbs on the UV exposed areas. The wells with the collagen patterns were incubated for 10 mins with 1µM solutions of H_{47N}, $H_{47Y<R}$, H_{47Kdel}, $H_{47Y<ONBY}$ or EGFP in PBS. For *in situ* photo activation of $H_{47Y<ONBY}$, the well filled with $H_{47Y<ONBY}$ was irradiated 10 s with a PRIMO unit at 365 nm and 50% intensity. Fluorescence imaging was done using a Nikon Ti-Eclipse microscope. All the experiments were done in triplicate.

Native PAGE Western Blot

3mg/mL rat tail collagen Type 1 (Fischer Scientific) was diluted in sterile D/W to a concentration of 200ug/mL. H_{47N}, $H_{47Y<R}$, $H_{47Y<ONBY}$, $H_{47Y<ONBYhv}$, H_{47Kdel} and EGFP solutions at 1.2µM concentration were mixed with collagen solutions in 2:1 ratio. The samples were incubated overnight at 4°C. 20ul were run in Native PAGE 4-16% Bis-Tris Protein Gel (Invitrogen).The proteins were blotted on PVDF membrane for WB. Anti-Collagen Type I (RABBIT) Antibody - 600-401-103-0.1 (Rockland antibodies and assays), Secondary; Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 647(Invitrogen) in 1:500 dilution were used for WB. All the experiments were done in triplicate.

Fibrillation Assay

Collagen Type I solution (3mg/mL, Fisher Scientific) was diluted to 0.4 mg/ml with minimal essential medium (MEM) Buffer (1x) and pH was adjusted to 7.5 with 1 M sodium hydroxide (Sigma-Aldrich). This process has to be done within 4 min to prevent premature polymerization.[4] The collagen solution was mixed with 1.2 mM solutions of H_{47N}, EGFP, $H_{47Y<R}$, $H_{47Y<ONBY}$ or $H_{47Y<ONBYhv}$ in MEM at molar ratio 1:2. The final concentration of collagen was 0.2 µg/ml(0.6 µM). A 0.2 µg/ml collagen solution was also used as a positive control. 100µl of the Col/Hsp47 variant mixture were transferred to a precooled 96 well plate and absorbance at 600 nm was recorded with a Spectra Max UV plate reader during 40 min. The optical density of the Collagen solution at 600 nm was measured under different conditions. This wavelength

does not interfere with the UV spectrum of ONBY. The $H_{47Y<ONBY}$ solution was irradiated at 365 nm for 5 mins for activation. All the experiments were done in triplicate.

KDEL mediated delivery of Hsp47 variants to fibroblasts

L929 fibroblasts and Mouse Embryonic Fibroblast (MEF) were seeded on 12 well µ-Slide Angiogenesis (Ibidi) with DMEM GlutaMax and RPMI GlutaMax medium containing 10% fetal bovine serum (FBS; Gibco), ascorbic acid phosphate, and antibiotics (20K cells per well). After 30 mins cells were incubated with 0.3 µM solution of Hsp47 variants in DMEM medium. After 3 hours incubation the medium was removed and cells were washed once with sterile Assay buffer (1X). Dual staining was prepared by mixing 1 µl of ER tracker (ER Staining Kit - Red Fluorescence - Cytopainter (ab139482)) with 1 µl of DAPI in 1 ml of ER Assay buffer (1X) provided in the kit. The cells were incubated with 60ul of Dual staining solution per well at 37°C for ½ hr. Cells were washed with Assay buffer (1X) once. Cells were fixed with 4% PFA for 10 mins. Washing was performed 3 times with Assay buffer (1X). All the experiments were done in triplicate.

Sirus Red Assay for quantification of Collagen deposition and Immunostanining:

Hsp47+/+ and Hsp47-/- MEFs derived from Lethal Mouse Embryos were gifted by Prof. Dr. Kazuhiro Nagata, Kyoto Sangyo University, Japan[5]. Cells were cultured in 24 well plate with high glucose DMEM Glutamax (Gibco) containing 10% FBS (Gibco), w/o or w/ ascorbic acid phosphate (0.1mM) (Ascorbate), and antibiotics for 24 hours (25K cells per well). Cells were incubated with 0.3µM solutions of Hsp47 variants or EGFP in medium for 3 hrs, followed by medium exchange w/ or w/o ascorbate, and cultured for additional 24 hrs. After PBS washing, cells were fixed using Bouin solution (75% picric acid, 10% formalin, and 5% acetic acid) (Sigma HT10132). Collagen deposited in the wells was stained by incubation with 0.1% Sirius red in picric acid (ab150681) for 1 hr and washing with 0.01 N HCl and 0.1 N NaOH[5e]. Collagen deposition was quantified at 570 nm using a Biolumin960k spectrophotometer. Results were normalized by taking Hsp47 (+/+) as 1. Cell Counting was performed using Image J with references to DAPI stained cells. All the analysis was performed in triplicate.Hsp47+/+ and Hsp47-/- cells (25K cells per well) were cultured in 24 well plates with high glucose DMEM Glutamax (Gibco) containing 10% FBS (Gibco), 0.1 mM ascorbate, and antibiotics. After 24 hrs, cells were incubated with 0.3µM solution of H_{47N}, $H_{47Y<ONBY,}$ H_{47Kdel} or EGFP in DMEM medium for 3 hrs. Afterwards the solution was exchanged by DMEM Glutamax with ascorbate and cells were cultured for further 24 hrs. The wells containing $H_{47Y<ONBY}$ were irradiated at 365 nm using PRIMO Nikon Microscope with 50% intensity for 30 seconds, 1 hour after medium exchange. After 24 hours cells were fixed and immuno stained against collagen with Primary; Rabbit polyclonal anti-type I collagen, 600-401-103-0.1 (Rockland; dilution 1:200), Secondary; Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 647, A-21245 (dilution 1:200) and stained Nucleus with DAPI (Thermofisher). Microscopic images were taken with Nikon Ti-Eclipse microscope. All the experiments were done in triplicates.

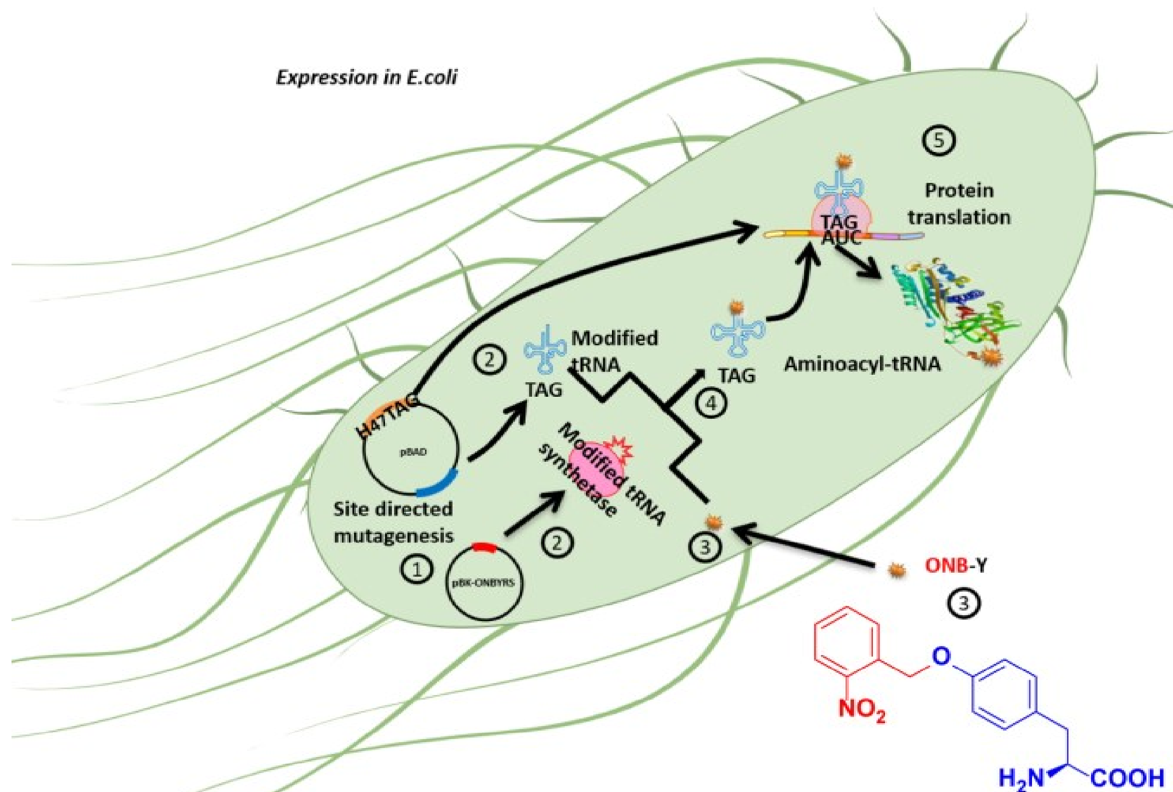


Figure S1. A. Schematic of synthesis of $H_{47Y<ONBY}$1. Co transformation of Hsp47383TAGpBAD and PBKONBYRS, 2. synthesis of orthogonal modified tRNA and tRNA synthetase, 3.uptake of ONBY by the *E.coli*,

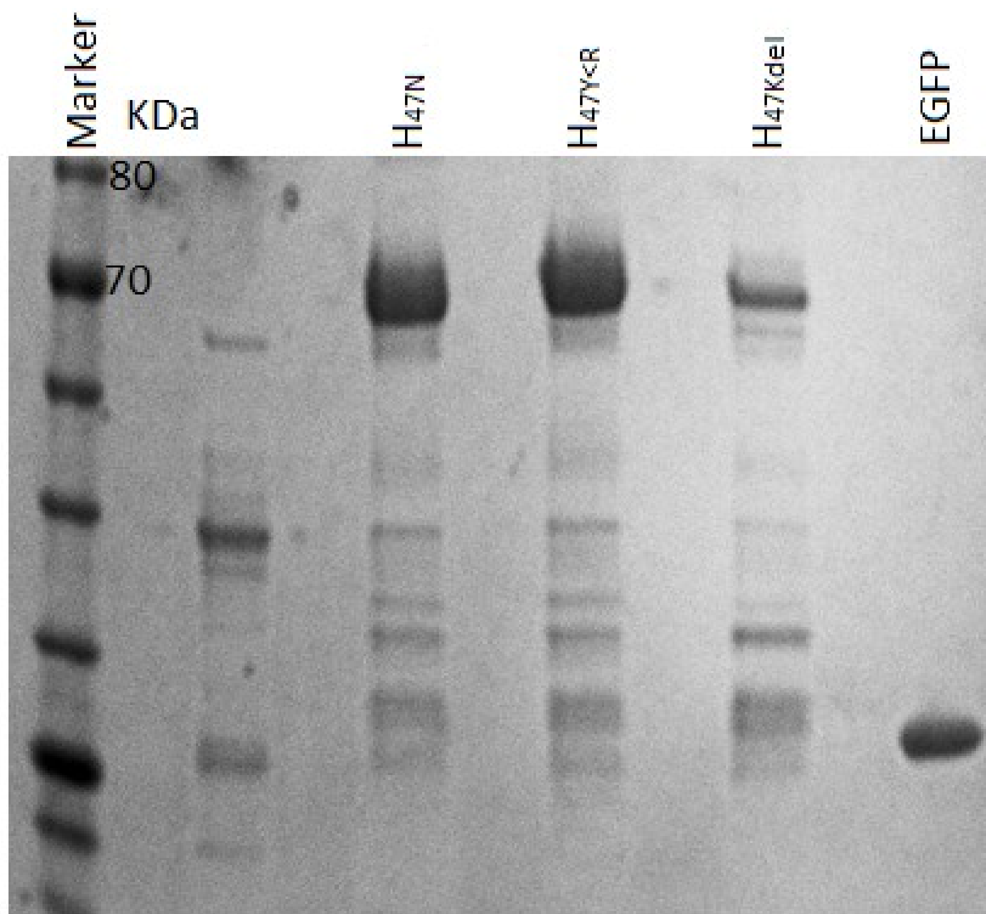


Figure S2. 12 % SDS PAGE gel of Hsp47 variants.

Table S1.Yield of the synthesis of Hsp47 mutants calculated from absorbance measurement using Nano drop UV spectrophotometer.

Sr.No	Title	Detail Plasmid & Genetic Construct	Yield (200mL culture)
1	H_{47}	cHsp47strepPJexp411	183.26ng (with impurities)
2	H_{47N}	EGFPHsp47strepTagpeT28a EGFPHsp47strepTagpBAD	207.8µg 65.98 ng
3	$H_{47Y<ONBYhv}$	EGFPHsp47strepTag383TAGMutpBAD	43.56 ng
4	H_{47Kdel}	EGFPHsp47strepTag C terminal KDEL Deletion peT28a	65.98ng
5	$H_{47Y<R}$	EGFPHsp47strepTag MutY383R peT28a	45.85 ng
6	EGFP	EGFPpeT28a	359.95 ng

Table S2.Primers used for developing $H_{47YN,}$ $H_{47Y<ONBY}$ and other mutants.

Sr.No	Title	Detail Plasmid & Genetic Construct	Primers
1	H_{47N}	EGFPHsp47strepTagpeT28a	ttaGAATTCATGCTGAGCCCGAAAGCC
			tttCTCGAGTTATTTCTCAAATTGCGGGTGG
		EGFPHsp47strepTagpBAD	tttCCATGGTGAGCAAGGGCGAG
			tttCTCGAGTTATTTCTCAAATTGCGGGTGG
2	$H_{47Y<ONBYhv}$	EGFPHsp47strepTag383TAGMutpBAD	AGCTGTTTTagGCGGATCATCCG
			TCGGGGAACGCAGCTCTT
3	H_{47Kdel}	EGFPHsp47strepTag C terminal KDEL Deleted peT28a	CTGGAATCCGCTTGGAGC
			GTCGCCCTTTGGACGGAC
4	$H_{47Y<R}$	EGFPHsp47strepTag MutY383R peT28a	GAAGCTGTTTcgCGCGGATCATCC
			GGGGAACGCAGCTCTTCA
5	EGFP	EGFPpeT28a	CTGGAATCCGCTTGGAGC
			GTCGCCCTTTGGACGGAC

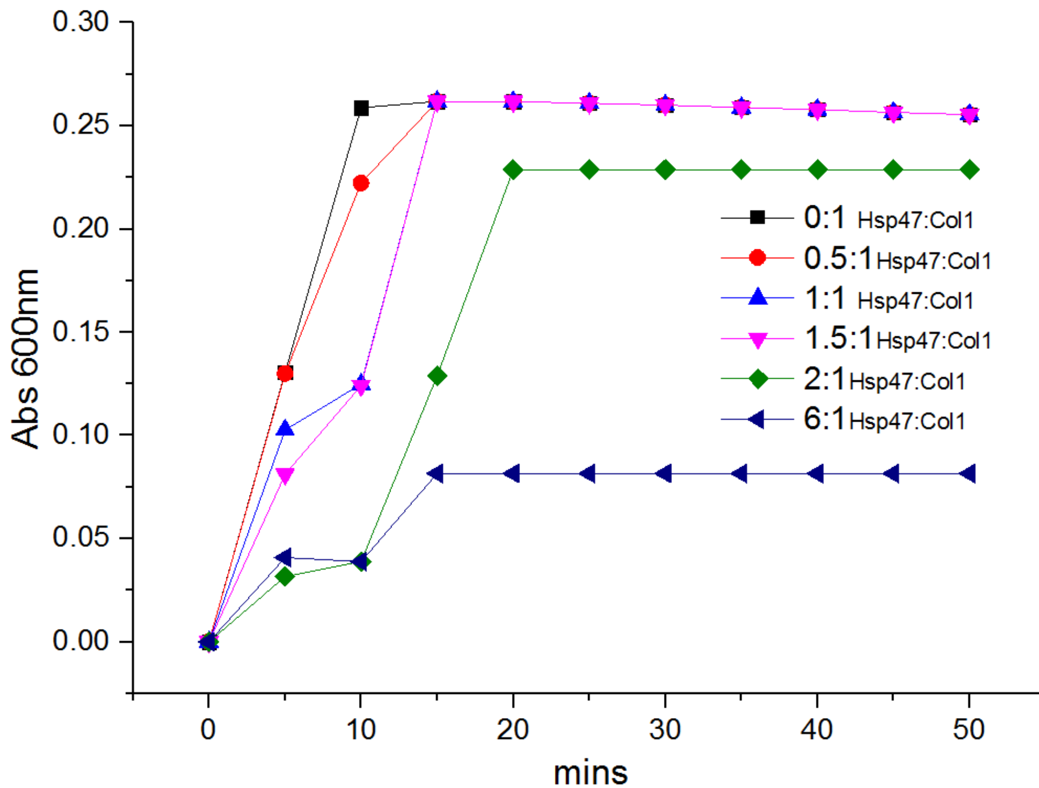
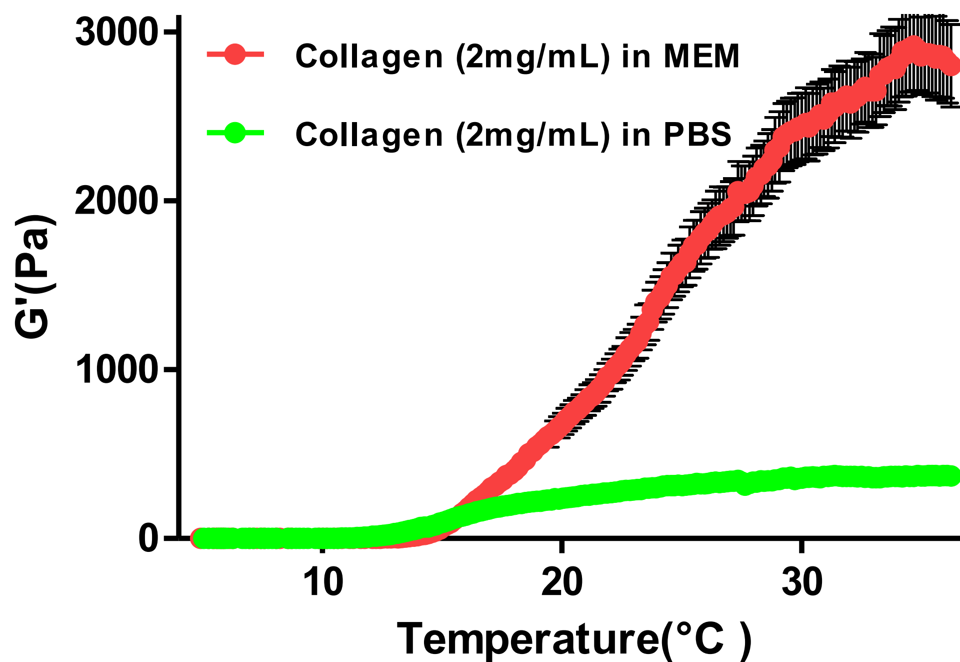


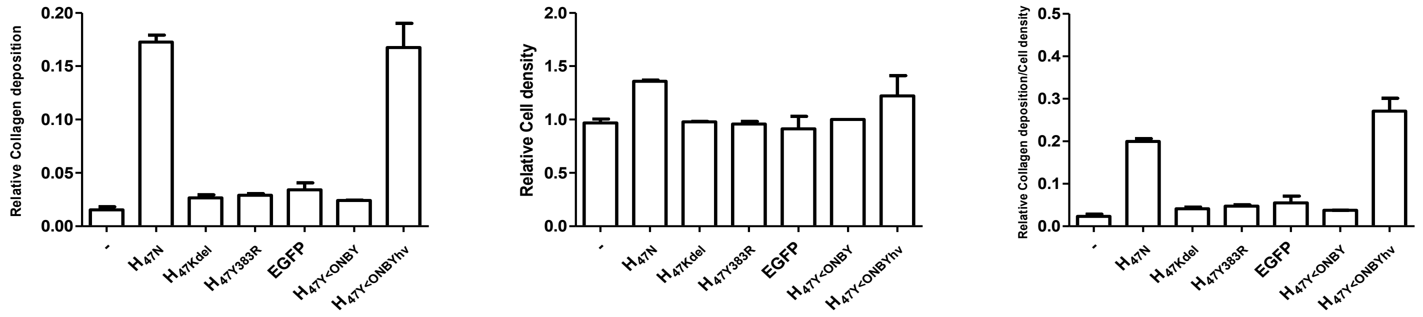
Figure S3. Turbidity measurements of Collagen/Hsp47 mixtures of different ratios. The increase in absorbance at 600nm reflects the formation of fibrils. Rat tail collagen (3mg/mL) was diluted to 0.4 mg/ml by addition of MEM Buffer (1x) and was adjusted to pH to 7.5 with sodium hydroxide within 4 min to prevent premature polymerization[4].The 0.4 mg/ml of collagen solution was mixed with H_{47N} at molar ratios of 0.5:1 (E-H_{47N} (0.3uM): Collagen (0.6uM)), 1:1 (H_{47N} (0.6uM): Collagen (0.6uM)), 2:1 (E-H_{47N} (1.2uM): Collagen (0.6uM)) and 6:1(H_{47N} (3.6uM): Collagen (0.6uM)) to a final concentration of 0.2 µg/ml. A 0.2 µg/ml collagen solution was used as a positive control. 75µl of each sample were transferred to a plate reader and absorbance was measured at 600 nm for 50 min at 34 ºC. The experiments were performed in triplicate.



Figur S4. Rheology measurements of collagen solutions during fibrillation in MEM and PBS. Rat tail collagen (3mg/mL) was diluted to 2 mg/mL concentration with MEM or PBS and was adjusted to pH to 7.5 with sodium hydroxide within 4 min. A DHRIII Rheometer (TA Instruments) was used for the measurements. 50 µl of the collagen solution were placed between two parallel plates of 8 mm diameter cooled at 4°C. The shear moduli (G') were measured at frequency ω - 30-0.03 rad/s while temperature was increased to 40°C at 0.1°C/min.

Hsp47 (-/-) w/o ascorbate   Hsp47 (-/-) w/ ascorbate

A



B

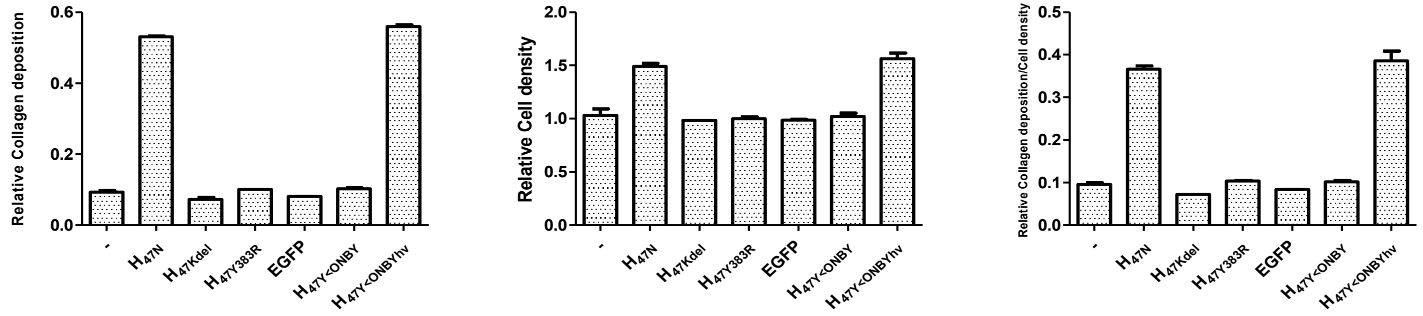


Figure S5. Relative collagen deposition and Relative Cell Density of MEF Hsp47 -/- in absence (A) and presence of ascorbate (B) with Hsp47 variants. Photo activation of $E\text{-}H_{47Y<ONBY}$ was done *in situ* by irradiating UV light for 30 sec using Nikon Ti-Eclipse microscope at 365nm wavelength.

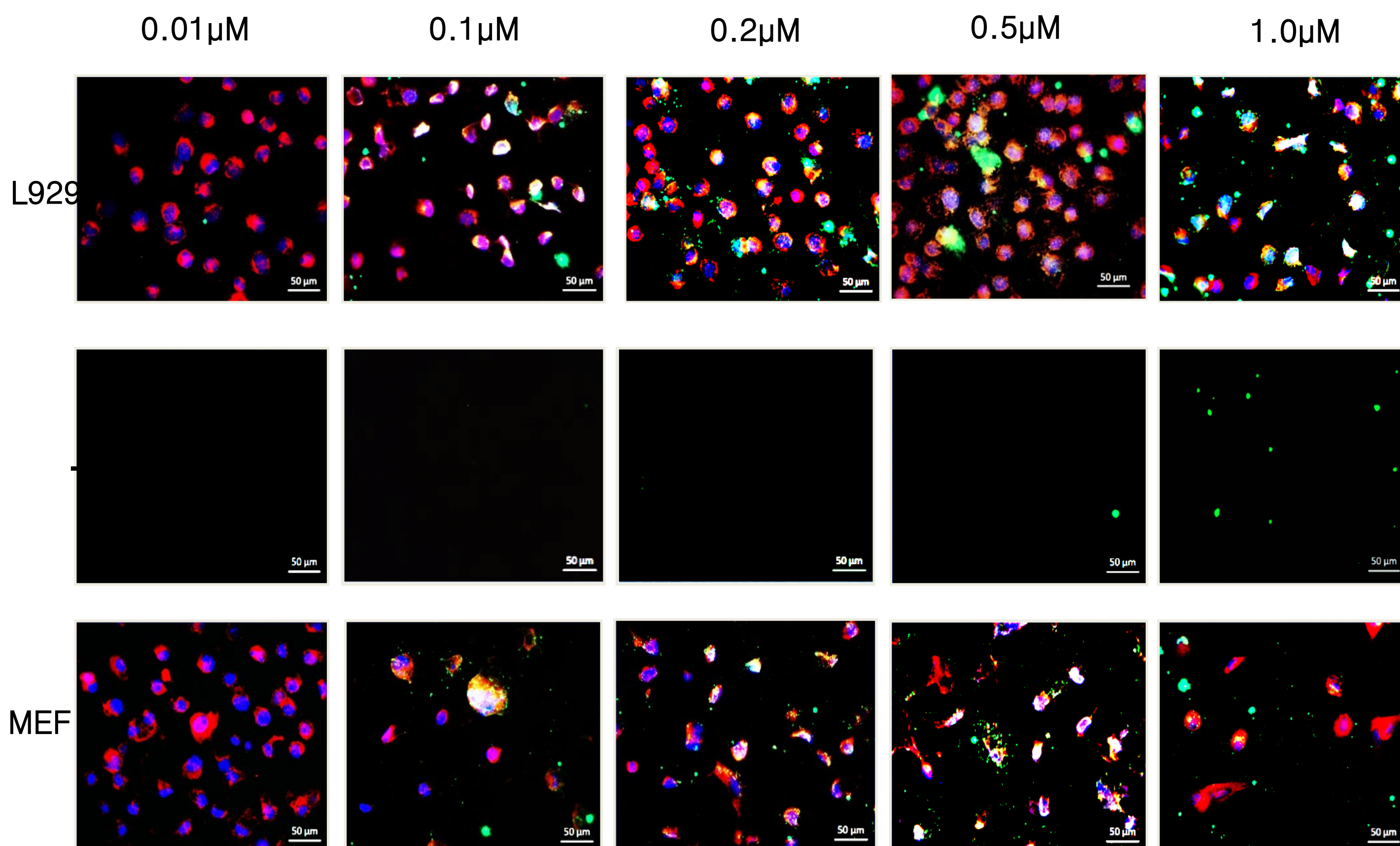


Figure S6. Fluorescence microscopy images of L929 and MEFs cells after incubation with different concentration of E-H_{47} from 0.01 to 1 μm during 3 hours, washing and fixation. (Blue: DAPI (Nucleus), Green: EGFP Hsp47, Red: Rhoda mine labeled ER). The images were taken using a Zen Observer Video Microscope (Zeiss).

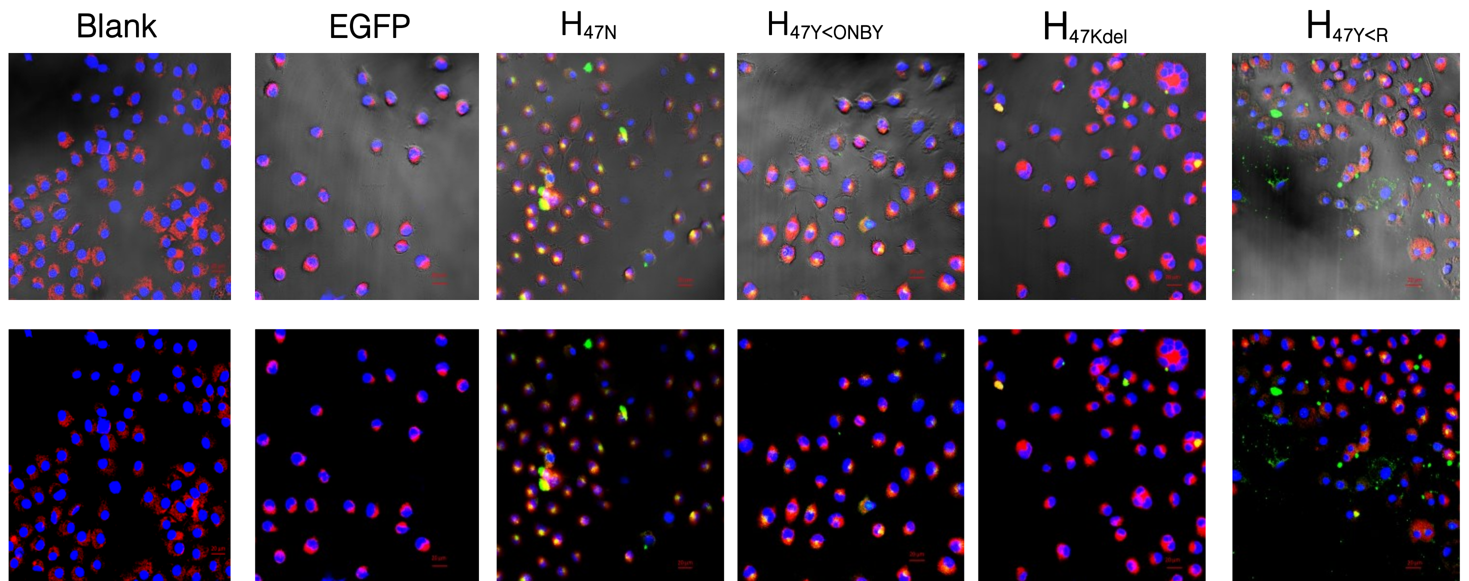


Figure S7.Confocal microscopy images of L929 cells after incubated with EGFP, $H_{47,}$ $H_{47Y<ONBY,}$ $H_{47Y<R}$ and H_{47Kdel}.Fixed and stained with Blue: DAPI (Nucleus), Green: EGFP Hsp47, Red: Rhoda mine labeled ER. Scale: 20μm

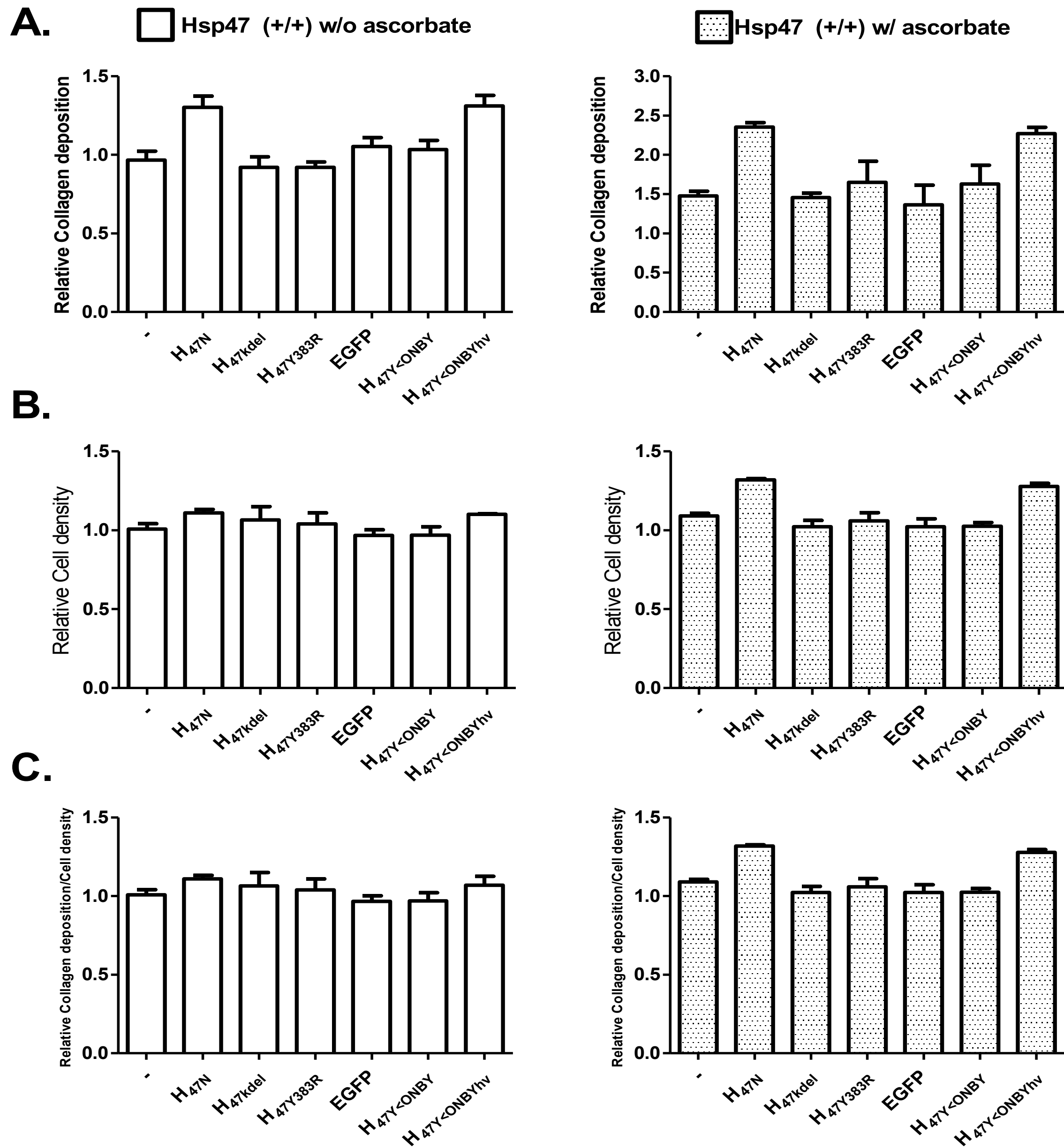


Figure S8. Relative collagen deposition, Relative Cell Density and Relative Collagen Production per Cell density of Hsp47 +/+ in absence and presence of ascorbate with different Hsp47 mutants. Collagen deposition was quantified using Sirus Red Assay. Cell density was counted using DAPI staining of the nucleus and Image J software. Photo activation of $H_{47Y<ONBY}$ was done *in situ* by 30 sec exposure at 365 nm using PRIMO Unit in Nikon Ti-Eclipse microscope at 50% illumination intensity.

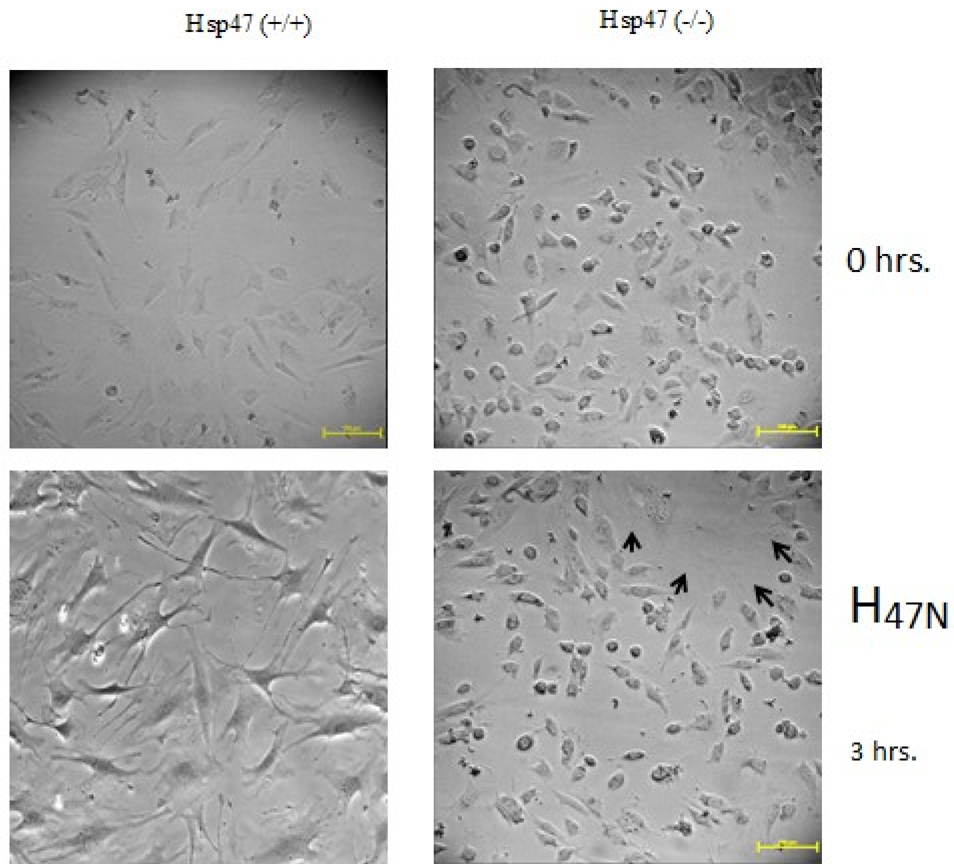


Figure S9. 20X Bright field images of Hsp47 (+/+) and Hsp47 (-/-) cells showing improvement shown with the small arrows in morphology after delivery of E-H_{47} in 3 hours

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PileUp
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 Name: Melonfly oo  Len:  421  Check:  5970  Weight:  10.0
 Name: ChannelCatfish oo  Len:  421  Check:  2938  Weight:  10.0
 Name: Amazonaparrot oo  Len:  421  Check:  2619  Weight:  10.0
 Name: Brandtsbat oo  Len:  421  Check:  2759  Weight:  10.0
 Name: Greenseaturtle oo  Len:  421  Check:  8976  Weight:  10.0
 Name: Rockdove oo  Len:  421  Check:   557  Weight:  10.0
 Name: Squerrillikeanimal oo  Len:  421  Check:  3579  Weight:  10.0
 Name: Yak oo  Len:  421  Check:   940  Weight:  10.0
 Name: Blackfruitbat oo  Len:  421  Check:  2467  Weight:  10.0
 Name: Nakedmolerat oo  Len:  421  Check:   796  Weight:  10.0
 Name: ChineseHamester oo  Len:  421  Check:  3110  Weight:  10.0
 Name: Damaralandmolerat oo  Len:  421  Check:  2798  Weight:  10.0
 Name: Mediterraneanfruitfly oo  Len:  421  Check:  3130  Weight:  10.0
 Name: Australian oo  Len:  421  Check:  6134  Weight:  10.0//

Homo    MRSLLLLSAF CLLEAALAAE VKKPAAAAAP GTAEKLSPKA ATLAERSAGL
Rat     .MRSLLLGTL CLLAVALAAE VKKPVEATAP GTAEKLSSKA TTLAERSTGL
Mus     .MRSLLLGTL CLLAVALAAE VKKPLEAAAP GTAEKLSSKA TTLAERSTGL
Cani    MRFLLLLNTC CLLAVVLAAE VKKPAAAAAP GSAEKLSPKA ATLAERSAGL
Gall    .......... ...MQIFLVL ALCGLAAAVP SEDRKLSDKA TTLADRSTTL
Zeb     .......... ...MWVSSLI ALCLLAVAVS GEDKKLSTHA TSMADTSANL
Gol     .......... ...MLVSSVV LLCLLATVSG ..DKALSSHA SILADNSANF
Mel     .......... .......... ...MEALKIT YKLERQFLVK FLFVLGATAL
Chan    .......... ...MWVKFLV GLCLLASVGA ..DKKLSSHA TILADNSANL
Amaz    .......... ...MWIILVL ALCGLAAAVP SEDRKLSDKA TTLADRSTTL
Bran    MRSLLLTSAF CLLAMALAAE VKKPAAPAAP GTAEKLSPKA TTLAERSAGL
Gree    .......... ...MWVTNLL ALCALVAAVP SEDKKLSDKA AALADRSATL
Roc     .......... ....MWIILV LALCGLAAAV PSEDRKLSDK ATTLADRSTT
Sque    MRSLLLLSTF CLLALAG... .......... .VLAAELSPK AATLAERSAG
Yak     MRALLLISTI CLLARALAAE VKKPAAAAAP .GTAEKLSPK AATLAERSAG
Blac    MRSLLLTSTF CLLAITLAAE VKKPAVAAAA PGTGEKLSPK AATLAERSAG
Nake    MRCLLLLGTF SLLAVALAAE VKKPAAAAAP G.TAEKLSSK AATLAERSAG
Chin    .MRSLLLASF CLLAVALAAE VKKPVEAAAP G.TAEKLSSK ATTLAERSTG
Dama    MRCLLLLGTF SLLAVALAAE VKKPAAVAAP G.TAEKLSSK AATLAERSAG
Medi    .......... .......... ....MMAYTY RHKLQIYFIA QILCTVWLTA
Aust    .......... .....MGMKL ILLSLLICVV KSEPVLLKEQ GPILGDSTVN

Homo    AFSLYQAMAK DQAVENILVS PVVVASSLGL VSLGGKATTA SQAKAVLSAE
Rat     AFSLYQAMAK DQAVENILLS PLVVASSLGL VSLGGKATTA SQAKAVLSAE
Mus     AFSLYQAMAK DQAVENILLS PLVVASSLGL VSLGGKATTA SQAKAVLSAE
Cani    AFSLYQAMAK DQAVENILLS PVVVASSLGL VSLGGKATTA SQAKAVLSAE
Gal     AFNLYHAMAK DKNMENILLS PVVVASSLGL VSLGGKATTA SQAKAVLSAD
Zeb     AFNLYHNVAK EKGLENILIS PVVVASSLGM VAMGSKSSTA SQVKSILKAD
Gol     AFNLYHNLAK EKDIENIVIS PVVVASSLGL VALGGKSNTA SQVKTVLSAT
Mel     AYGQDDGFAQ D...DNVYSW YILDVTRILQ NAESNIVVSP SNIRALLKTP
Chan    AFDLYHNMAK EKDMENILIS PVVVASSLGL VALGGKASTA SQVKTVLSGN
Amaz    AFNLYXAMAK DKNMENILLS PVVVASSLGL VSLGGKATTA SQAKAVLSAD
Bran    AFSLYQAMAK DQAVENILLS PVVVASSLGL VSLGGKATTA SQAKAVLSAE
Gree    AFNLYHTMAK DKNMENILVS PVVVASSLGL VSLGGKATTA SQAKAVLSAD
Roc     LAFNLYHAMA KDK..NMENI LLSPVVVASS LGLVSLGGKA ATASQAKAVL
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Sque     LAFSLYQAMA KDQ..AVENI LLSPVVVASS LGLVSLGGKA ATASQAKAVL
Yak      LAFSLYQAMA KDQ..AVENI LLSPVVVASS LGLVSLGGKA ATASQAKAVL
Blac     LAFSLYQAMA KDQ..AVENI LLSPVVVASS LGLVSLGGKA TTASQAKAVL
Nake     LAFSLYQAMA KDQ..AVENI LLSPVVVASS LGLVSLGGKA ATASQAKAVL
Chin     LAFSLYQAMA KDQ..AVENI LLSPLVVASS LGLVSLGGKA TTASQAKAVL
Dama     LAFSLYQAMA KDQ..AVENI LLSPVVVASS LGLVSLGGKA ATASQAKAVL
Medi     TVCGESDKYS EDEVVNENNV LAWYILDVSQ ILQNSKVNTI LSP...MNLL
Aust     LGLSLYQTMI KDQKLRSQNL LFSPVVVASS LGVMSMGAKD KTAKQVKSLL

Homo     QLRDEEVHAG LGELLRSLSN STARNVTWKL GSRLYGPSSV SFADDFVRSS
Rat      KLRDEEVHTG LGELLRSLSN STARNVTWKL GSRLYGPSSV SFADDFVRSS
Mus      KLRDEEVHTG LGELLRSLSN STARNVTWKL GSRLYGPSSV SFADDFVRSS
Cani     QLRDEEVHAG LGELLRSLSN STARNVTWKL GSRLYGPSSV SFAEDFVRSS
Gal      KLNDDYVHSG LSELLNEVSN STARNVTWKI GNRLYGPASI NFADDFVKNS
Zeb      ALKDEHLHTG LSELLTEVSD PQTRNVTWKI SNRLYGPSSV SFAEDFVKNS
Gol      TVKDEQLHSG LSELLTEVSN STARNVTWKI SNRLYGPSSV SFVDNFLKSS
Mel      PSMN..LRFG FDEKTMLG.. ...QNVIFAE SNMILNNP.. .......DTL
Chan     KVKDENLHSG LAELLSEVSN PKERNVTWKI TNRLYGPSSV SFSEDFVKNS
Amaz     KLNDDYVHSG LSELLNEVSN STARNVTWKI GNRLYGPASI NFADDFVKNS
Bran     QLRDEEVHAG LGELLRSLSN STARNVTWKL GSRLYGPSSV SFAEDFVRSS
Gree     KLNDDYIHGG LSELLNEVSN STARNVTWKL GNRLYGPSSI SFAEDFVKSS
Roc      SADKLNDDYL HSGLSELLNE VSNSTARNVT WKIGNRLYGP ASINFADDFV
Sque     SAEQLRDEEV HAGLGELLRS LSNSTARNVT WKLGSRLYGP SSVSFAEDFV
Yak      SAEQLRDDEV HAGLGELLRS LSNSTARNVT WKLGSRLYGP SSVSFAEDFV
Blac     SAEQLRDEEV HAGLGELLRS LSNNTARNVT WKLGSRLYGP SSVSFADDFV
Nake     SAEQLRDEEV HAGLGELLRS LSNSTARNVT WKLGSRLYGP SSVSFADDFV
Chin     SAEKLRDEEV HTGLGELLRS LSNSTARNVT WKLGSRLYGP SSVNFAEDFV
Dama     SAEQLRDEEV HTGLGELLRS LSNSTARNVT WKLGSRLYGP SSVSFADDFV
Medi     KTPAIVDLRF GADN.EMEGI NMILAESRRS LSRP...... ..........
Aust     NIN.LNDDTL HPAFSELLNE VSNETARNTT WKIGNCLYAP TSVNVRDDFV

Homo     KQHYNCEHSK INFRDKRSAL QSINEWAAQT TDGKLPEVTK DVERTDGALL
Rat      KQHYNCEHSK INFRDKRSAL QSINEWASQT TDGKLPEVTK DVERTDGALL
Mus      KQHYNCEHSK INFRDKRSAL QSINEWASQT TDGKLPEVTK DVERTDGALL
Cani     KQHYNCEHSK INFRDKRSAL QSINEWAAQT TDGKLPEVTK DVERTDGALL
Gal      KKHYNYEHSK INFRDKRSAL KSINEWAAQT TDGKLPEVTK DVEKTDGALI
Zeb      KKHYNYEHSK INFRDKRSAI NSINEWAAKT TDGKLPEITK DVKNTDGAMI
Gol      KKHYNCEHSK INFRDKRSAV KAINDWASKS TDGKLPEVTK DVEKTDGAMI
Mel      EWFYDCKIQE TSFTDKKKLI TSINDWSENI ADQTILKSSE MILKEENLQV
Chan     KKHYKYEHAK INFRDKKSAV NAINEWASKS TDGKLPEVTK DVEKTDGAMI
Amaz     KKHYNYEHSK INFRDKRSAL KSINEWAAQT TDGKLPEVTK DVEKTDGALI
Bran     KLHYNCEHSK INFRDKRSAL QSINEWASQT TDGKLPEVTK EVERTDGALL
Gree     KKHYNYEHSK INFRDKRSAL KSINEWASQT TNGKLPEVTT NVEKTDGALI
Roc      KNSKKHYNYE HSKINFRDKR SALKSINEWA AQTTDGKLPE VTKDVEKTDG
Sque     RSSKQHYNCE HSKINFRDKR SALQSINEWA AQTTGGKLPE VTSDVERTDG
Yak      RSSKQHYNCE HSKINFRDKR SALQSINEWA AQTTDGKLPE VTKDVERTDG
Blac     RSSKQHYNCE HSKINFRDKG SALQSINEWA AQTTDGKLPE VTKEVERTDG
Nake     RSSKQHYNCE HSKINFRDKR TALQSINEWA AQTTDGKLPE VTKDVERTDG
Chin     HSSKQHYNCE HSKINFRDKR SALQSINEWA SQTTDGKLPE VTKDVERTDG
Dama     RSSKQHYNCE HSKINFRDKR SALQSINEWA AQTTDGKLPE VTKDVERTDG
Medi     ENVEMFYNTK IQEISFADTA NHLVMINDWG KRVTNEEFPK LIESNSSLKN
Aust     QKTKTHYKYD HSQINFKDQR SALRSINQWA SQATEGKLSE ITAALSSTDG

Homo     VNAMFFKPHW DEKFHHKMVD NRGFMVTRSY TVGVMMMHRT GLYNYYDDEK
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```
Rat      VNAMFFKPHW DEKFHHKMVD NRGFMVTRSY TVGVTMMHRT GLYNYYDDEK
Mus      VNAMFFKPHW DEKFHHKMVD NRGFMVTRSY TVGVTMMHRT GLYNYYDDEK
Cani     VNAMFFKPHW DEKFHHKMVD NRGFMVTRSY TVGVTMMHRT GLYNYYDDEK
Gal      VNAMFFKPHW DEKFHHKMVD NRGFMVTRSY TVGVPMMHRT GLYNYYDDEA
Zeb      VNAMFFKPHW DEKSHHKMVD NRGFLVTRSH TVSVPMMHRT GIYGFYEDTE
Gol      INAMFYKPHW DEQFHHKMVD NRGFLVHRSY TVSVPMMHRT GIYGLFDDTT
Mel      LILNMLNFKE TLQINFKYTL NATFHERPDS TIVLPAVETT EYLKYLDSQI
Chan     INAIFYKPHW DEQFHHQMVD NRAFLVHRSY TVSVPMMHRT GIYGFYDDTA
Amaz     VNAMFFKPHW DEKFHHKMVD NRGFMVSRSY TVGVPMMHRT GLYNYYDDET
Bran     VNAMFFKPHW DERFHHKMVD NRGFMVTRSY TVGVTMMHRT GLYNYYDDEK
Gree     VNAMFFKPHW EERFHHKMVD NRGFMVTRSY TVGVPMMHRT GLYNYFDDEA
Roc      ALIVNAMFFK PHWDEKFHHK MVDNRGFMVT RSYTVGVPMM HRTGLYNYYD
Sque     ALLVNAMFFK PHWDERFHHK MVDNRGFMVT RSYTVGVTMM HRTGLYNYYD
Yak      ALLVNAMFFK PHWDERFHHK MVDNRGFMVT RSYTVGVTMM HRTGLYNYYD
Blac     ALLVNAMFFK PHWDERFHHK MVDNRGFMVT RSYTVGVTMM HRTGLYNYYD
Nake     ALLVNAMFFK PHWDEKFHHK MVDNRGFMVT RSYTVGVTMM HRTGLYNYYD
Chin     ALLVNAMFFK PHWDEKFHHK MVDNRGFMVT RSYTVGVTMM HRTGLYNYYD
Dama     ALLVNAMFFK PHWDEKFHHK MVDNRGFMVT RSYTVGVTMM HRTGLYNYYD
Medi     LQVLILNMFH FVETLEINFK YTANLLFYIT PKQRTKVPAV ETTEYLKYLD
Aust     AFIINANYFK PHWDESFQQT MVDKRGFIIT RTHTVSIPMM HQIRLCNYYE

Homo       EKLQIVEMPL AHKLSSLIIL MPHHVEPLER LEKLLTKEQL KIWMGKMQKK
Rat        EKLQLVEMPL AHKLSSLIIL MPHHVEPLER LEKLLTKEQL KTWMGKMQKK
Mus        EKLQMVEMPL AHKLSSLIIL MPHHVEPLER LEKLLTKEQL KAWMGKMQKK
Cani       EKLQIVEMPL AHKLSSLIIL MPHHVEPLER LEKLLTKEQL KIWMGKMQKK
Gal        EKLQVVEMPL AHKLSSMIFI MPNHVEPLER VEKLLNREQL KTWASKMKKR
Zeb        NRFLIVSMPL AHKKSSMIFI MPYHVEPLDR LENLLTRQQL DTWISKLEER
Gol        NNLLVLDMAL AHKMSSIVFI MPYHVESLER VEKLLTRQQL NTWISKMEQR
Mel        LDAKILELPY SNG.YFMYII LPHTKQGVIE TINNLGYEQL TRIEWMMKER
Chan       NSFFVLEMPL AHKKSSVIFI MPYHVESLER LEKMLTRKQL DIWQSKMEQK
Amaz       EKLQVVEMPL AHKLSSMIFI MPNHVEPLER VEKLLNREQL KTWAGKMKKR
Bran       EKLQIVEMPL AHKLSSLIIL MPHHVEPLER LEKMLTKEQL KTWMGKMQKR
Gree       EKLQIVEMPL AHKLSSMIFI MPNHVEPLER VEKLLTREQL KTWIGKLKKR
Roc        DETEKLQVVE MPLAHKLSSM IFIMPNHVEP LERVEKLLNR EQLKTWAGKM
Sque       DEKEKLQIVE MPLAHKLSSL IIIMPHHVEP LERLEKLLTK EQLKTWMGKM
Yak        DEKEKLQMVE MPLAHKLSSL IIIMPHHVEP LERLEKLLTK EQLKVWMGKM
Blac       DEKEKMQIVE MPLAHKLSSL IILMPHHVEP LERLEKMLTK EQLKIWMGKM
Nake       DEKEKVQILE MPLAHKLSSL IILMPHHVEP LERLEKLLTK EQLKAWTGKL
Chin       DEKEKLQILE MPLAHKLSSL IILMPHHVEP LERLEKLLTK EQLKAWMGKM
Dama       DEKEKVQILE MPLAHKLSSL IILMPHHVEP LERLEKLLTK EQLKVWMGKM
Medi       SQMLDAKILQ LPYSNG.FSM YILLPHTKAG LNELLSILGF EQLKRLQWMM
Aust       DNANSLQVLE LPLSHKHSSM IFIMTKHIEP LARLEKLLTK EQLNTWIGKL

Homo       AVAISLPKGV VEVTHDLQKH LAGLGLTEAI DKNKADLSRM SGKKDLYLAS
Rat        AVAISLPKGV VEVTHDLQKH LAGLGLTEAI DKNKADLSRM SGKKDLYLAS
Mus        AVAISLPKGV VEVTHDLQKH LAGLGLTEAI DKNKADLSRM SGKKDLYLAS
Cani       AVAISLPKGV VEVTHDLQKH LAGLGLTEAI DKNKADLSRM SGKKDLYLAS
Gal        SVAISLPKVV LEVSHDLQKH LADLGLTEAI DKTKADLSKI SGKKDLYLSN
Zeb        AVAISLPKVS MEVSHDLQKH LGELGLTEAV DKPKADLSNI SGKKDLYLSN
Gol        AVAVSLPKVS VEVSHDLQKH LTELGLTEAV DKAKADLSNI SGKKDLYLSN
Mel        RVNVVMPTFK YHFITNMREH IQKN....SA HRFDVDFEPA FG.VETKKIN
Chan       AVAVSLPKIS MEVSHNLQKY LGELGVTEAV DKTKADLSNI SGKKDLYLAN
Amaz       SVAISLPKVV LEVSHDLQKH LADLGLTEAI DKTKADLSKI SGKKDLYLSN
Bran       AVAISLPKGV VEVTHDLQKH LAGLGLTEAI DKNKADLSRM SGKKDLYLAS
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Gree       AVAISLPKVS LEVSHDLQKH LADLGLTEAM DKNKADLSKI SGKKDLYLSN
Roc        KKRSVAISLP KVVLEVSHDL QKHLADLGLT EAIDKTKADL SKISGKKDLY
Sque       QTKAVAISLP KGVVEVTHDL QKHLATLGLT EAIDKNKADL SRMSGKKDLY
Yak        QKKAVAISLP KGVVEVTHDL QKHLAGLGLT EAIDKNKADL SRMSGKKDLY
Blac       QKKAVAISLP KGVVEVTHDL QKHLAGLGLT EAIDKNKADL SRMSGKKDLY
Nake       QKKAVAISLP KGVVEVTHDL QKHLAGLGLT EAIDKNKADL SRMSGKKDLY
Chin       QKKAVAISLP KGVVEVTHDL QKHLAGLGLT EAIDKNKADL SRMSGKKDLY
Dama       QKKAVAISLP KGVVEVTHDL QKHLAGLGLT EAIDKTKADL SRMSGKKDLY
Medi       EVRRVNVLMP TFKYSFITNL KEDILDK... .SDHRFDSDF ENSFSKEKDF
Aust       ERHTVSVSLP KVNLEVSHDL QKYLQELGLT EAVDKNKADF SGITGKKNLH

Homo       VFHATAFELD TDGNPFDQDI YGREELRSPK LFYADHPFIF LVRDTQSGSL
Rat        VFHATAFEWD TEGNPFDQDI YGREELRSPK LFYADHPFIF LVRDNQSGSL
Mus        VFHATAFEWD TEGNPFDQDI YGREELRSPK LFYADHPFIF LVRDNQSGSL
Cani       VFHATAFEWD TEGNPFDQDI YGREELRSPK LFYADHPFIF LVRDTQSGSL
Gal        VFHAAALEWD TDGNPYDADI YGREEMRNPK LFYADHPFIF MIKDSKTNSI
Zeb        VFHASSLEWD TEGNPFDPSI FGSEKMRNPK LFYADHPFIF LVKDNKTNSI
Gol        VFHASAMEWD TEGNPPDTSI YGTDKLKTPK LFYADHPFIF LVKDKKTNSI
Mel        IFQTTLVQFD GSG.RARVED YEKIRTTKYE RFHVDRPFAF YIKEKSTGRI
Chan       VFHASAFEWD IAGNPADTSI FGTDKVKNPK LFYVDHPFIF LVKDKSTGSI
Amaz       VFHAAALEWD TEGNPYDADI YGREEMRNPK LFYADHPFIF MIKDSKTNSI
Bran       VFHATAFEWD TEGNPFDQDI YGREELRSPK LFYADHPFIF LVRDSQTGSL
Gree       VFHAAALEWD TEGNPFDADI YGREEMRNPK LFYADHPFVF VIKDNKTNSI
Roc        LSNVFHATAL EWDTEGNPYD ADIYGREEMR NPKLFYADHP FIFMIKDNKT
Sque       LASVFHATAF ELDTEGNPFD QDIYGREELR SPKLFYADHP FIFLVRDTQS
Yak        LASVFHATAF EWDTDGNPFD QDIYGREELR SPKLFYADHP FIFLVRDTQS
Blac       LASVFHATAF EWDTEGNPFD QDIYGREELR SPKLFYADHP FIFLVRDSQS
Nake       LASVFHATAF EWDTEGNPFD QDIYGREELR SPKLFYADHP FIFLVRDTQS
Chin       LASVFHATAF EWDTDGNPFD QDIYGREELR SPKLFYADHP FIFLVRDNQS
Dama       LASVFHATAF EWDTEGNPFD QDIYGREELR SPKLFYADHP FIFLVRDTQS
Medi       LN.IFKVAAI SFNGTGKPRV EDYQNIR.SA KYEKFHVDRP FVYYIEN.KY
Aust       LSGMLHATAI DWDTEGNQFD QDWNSPEITK SAKVFYADHA YIFLIRDNKT

Homo                  LFIGRLVRPK GDKMRDEL
Rat                   LFIGRLVRPK GDKMRDEL
Mus                   LFIGRLVRPK GDKMRDEL
Cani                  LFIGRLVRPK GDKMRDEL
Gal                   LFIGRLVRPK GDKMRDEL
Zeb                   LFIGRLVRPK GDKMRDEL
Gol                   LFMGRLVQPK GDKMRDEL
Mel                   VCIGKVLNPV Q.......
Chan                  LFIGRLVRPK GEKMRDEL
Amaz                  LFIGRLVRPK GDKMRDEL
Bran                  LFIGRLVRPK GDKMRDEL
Gree                  LFIGRLVKPK GDKMRDEL
Roc                   NSILFIGRLV RPKGDKMRDE L
Sque                  GSLLFIGRLV RPKGDKMRDE L
Yak                   GSLLFIGRLV RPKGDKMRDE L
Blac                  GSLLFIGRLV RPKGDKMRDE L
Nake                  GSLLFIGRLV RPKGDKMRDE L
Chin                  GSLLFIGRLV RPKGDKMRDE L
Dama                  GSLLFIGRLV RPKGDKMRDE L
Medi                  GEIVCIGKVE NPEQ...... .
Aust                  NSILLIGRLV KPKSNDHDEL .
```

Figure S10. Multiple sequence alignment of Hsp47 showing conserved residues in different species. The KDEL sequence is highlighted in Green, active site 383 Y is highlighted in Blue, Histidine involved in protonation are highlighted in Red. RCL loop in Grey and Aspartic acid and Histidine are highlighted in Yellow and Pink.

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